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June 12, 2009

TSCA Confidential Business Information Center (7407M)
EPA East - Room 6428, Attn: Section 8(e) & FYI
U.S. Environmental Protection Agency
1200 Pennsylvania Avenue, NW
Washington, DC 20460-0001

Re: Submission To TSCA 8(e)/FYI Database Re: PFOA/PFOS

To TSCA 8(e)/FYI Database:

We are hereby providing the following information for inclusion in the TSCA 8(e)/ FYI databases with respect to PFOA/PFOS:

1. Fenton, S.E., et al., "Analysis of PFOA in Dosed CD-1 Mice Part 2: Disposition of PFOA in Tissues and Fluids From Pregnant and Lactating Mice and Their Pups," *Reprod. Toxicol.* (2009), doi:10.1016/j.reprotox.2009.02.012;

2. von Ehrenstein, O.S., et al., "Polyfluoroalkyl Chemicals in the Serum and Milk of Breastfeeding Women," *Reprod. Toxicol.* (2009), doi:10.1016/j.reprotox.2009.03.001; and

3. Hines, E.P., et al., "Phenotypic Dichotomy Following Developmental Exposure to Perfluorooctanoic Acid (PFOA) in Female CD-1 Mice: Low Doses Induce Elevated Serum Leptin and Insulin, and Overweight in Mid-Life," 304 *Molecular & Cellular Endocrinology* 97-105 (2009).

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Very truly yours,

Robert A. Bilott

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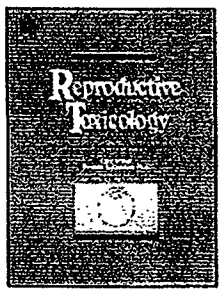

Title: Analysis of PFOA in Dosed CD-1 Mice Part 2: Disposition of PFOA in tissues and fluids from pregnant and lactating mice and their pups

Authors: Suzanne E. Fenton, Jessica L. Reiner, Shoji F. Nakayama, Amy D. Delinsky, Jason P. Stanko, Erin P. Hines, Sally S. White, Andrew B. Lindstrom, Mark J. Strynar, Syrago-Styliani E. Petropoulou

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Analysis of PFOA in Dosed CD-1 Mice Part 2: Disposition of PFOA in tissues and fluids from pregnant and lactating mice and their pups.

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Running title: PFOA disposition in lactation

Abbreviations

ANOVA	analysis of variance
BW	body weight
GD	gestational day
LOD	limit of detection
LOQ	limit of quantitation
MS	mass spectrometer
PFAA	perfluoroalkyl acid
PFOA	perfluorooctanoic acid
PFOS	perfluorooctane sulfonate
PND	postnatal day
SEM	standard error of the mean
UPLC	ultra performance liquid chromatography

Abstract

Previous studies in mice with multiple gestational exposures to perfluorooctanoic acid (PFOA) demonstrate numerous dose dependent growth and developmental effects which appeared to worsen if offspring exposed *in utero* nursed from PFOA-exposed dams. To evaluate the disposition of PFOA in the pregnant and lactating dam and her offspring, time-pregnant CD-1 mice received a single 0, 0.1, 1, or 5 mg PFOA/kg BW dose (N=25/dose group) by gavage on gestation day 17. Maternal and pup fluids and tissues were collected over time. Pups exhibited significantly higher serum PFOA concentrations than their respective dams, and their body burden increased after birth until at least 8 days old, regardless of dose. The distribution of milk:serum PFOA varied by dose and time, but was typically in excess of 0.20. These data suggest that milk is a substantial PFOA exposure route in mice and should be considered in risk assessment modeling designs for this compound.

Key words: PFOA; serum; amniotic fluid; urine; milk; mammary gland; dosimetry; disposition

1. Introduction

Perfluorooctanoic acid (PFOA) is a member of the perfluoroalkyl acid (PFAA) family of man-made, fluorinated organic compounds used in a number of consumer goods and industrial surfactants due to their grease and water-repellant properties. The use of PFAAs in many common applications, such as stain repellants for clothing, carpeting, and upholstery, and the stability of the carbon-fluorine bond have made them ubiquitous in the environment. The predominant route of exposure in North American and European consumers is likely oral intake, including drinking water, while inhalation and dermal absorption comprise routes of lesser exposure [1-5].

PFAAs are persistent, readily absorbed, not known to be metabolized, and are poorly eliminated, with half-lives in humans ranging from roughly 4-8 years [2-4]. In fact, the arithmetic and geometric mean half-lives of serum elimination, respectively, were 5.4 years [95% confidence interval (CI), 3.9-6.9] and 4.8 years (95% CI, 4.0-5.8) for PFOS; 8.5 years (95% CI, 6.4-10.6) and 7.3 years (95% CI, 5.8-9.2) for PFHS; and 3.8 years (95% CI, 3.1-4.4) and 3.5 years (95% CI, 3.0-4.1) for PFOA [4].

These characteristics led to increased concern for the potential health risks of PFAAs and a program to reduce product and emission content of PFOA and related chemicals was recently initiated [1]. PFAAs are continually detected worldwide in both human and wildlife samples [3, 6-9]. A recent analysis of American Red Cross blood donors indicated a reduction of 60% in blood perfluorooctane sulfonate (PFOS) and 25% in blood PFOA levels between the years 2000 and 2006[10]. However, while the production of and potential for human and wildlife exposures to certain PFAAs has been reduced in the US in recent years, it is not clear that perfluorinated compounds produced

in other countries will not continue to replace them in the US marketplace or in the contribution to worldwide exposure.

Much of the recent health effects research on PFOA in mice, commonly associated with gestational exposures of 0.01-5 mg PFOA/kg BW, has focused on developmental toxicities such as decreased maternal weight gain, reduced neonatal survival and body weight, as well as later life effects such as pubertal delays, mammary gland abnormalities, and excessive weight gain [2, 11-16]. Early postnatal adverse health observations prompted studies examining the effect of PFOA on maternal lactation and health effects of the nursing offspring. White et al. [14] described reduced epithelial differentiation on postnatal day (PND) 10 in mammary glands of CD-1 mouse dams exposed to 5 mg PFOA/kg BW from GD1-17, as well as delays in epithelial involution and alterations in milk protein gene expression on PND20. In addition, female offspring of exposed dams displayed stunted mammary epithelial branching and growth on PND10 and PND20. In a cross-foster study utilizing CD-1 mice, Wolf et al. [16] reported that although *in utero* exposure to 5 mg PFOA/kg BW from GD1-17 in the absence of lactational exposure was sufficient to induce postnatal body weight deficits and developmental delays, pup survival from birth to weaning was affected only in those both *in utero* and lactationally exposed. Furthermore, recent studies [15] have shown that unexposed neonates lactationally exposed to PFOA quickly developed mammary gland growth deficits and that control dams nursing *in utero*-exposed pups (dams exposed via pup grooming) demonstrated slowed differentiation of their own mammary glands that was evident in whole mount preparations of the tissue by the 5th day of lactation. These

results support a role for impaired lactational development and possibly a significant lactational transfer of PFOA in the observation of early growth effects.

The concern for potential prenatal and neonatal exposures in humans has been raised further by the detection of PFAAs in human breast milk and cord blood and the development-related outcomes associated with these observations. So et al. [17] indicated a range of 47-210 ng/L (0.047-0.21 ng/ml) PFOA in 19 samples of breast milk from Chinese women. PFOA was detected in only one of 12 human milk samples collected from 1996-2004 in Sweden at a concentration of 492 pg/ml (0.492 ng/ml; [18], and a mean of 43.8 pg/ml (0.044 ng/ml) was reported for 45 U.S. breast milk samples collected in 2004 [19]. Two studies recently determined a negative association between PFOA and growth indices in children with median cord serum levels of 1.6 ng/ml PFOA in the U.S. [20] and 5.6 ng/ml PFOA in Denmark [21]. While only one sample was found to contain PFOA in the Karrman et al. [18] study, these researchers reported a significant milk to serum correlation (r^2 = 0.7-0.8, p<0.05) for other PFAAs detected. Furthermore, Tao et al. [19] suggested that there may be preferential partitioning of PFOA to milk compared to other PFAAs and also indicated that women who were nursing for the first time exhibited 49% higher concentrations of PFOA in breast milk than women who had nursed previously, although inter-individual variation, daily milk output and milk protein concentration were not taken into consideration. The only study that has evaluated the distribution coefficient of PFCs between blood and milk in animal models was a pharmacokinetic study of placental and lactational transport of PFOA in rats[22]. Although female rats are known to have a serum PFOA half-life of only a few hours [23], unlike mice which have a ½-life of about 15 days [13], the study [22] indicated

concentrations in rat milk approximately 10 times less than that of maternal plasma and that the milk concentrations were generally of the same magnitude as the concentrations in pup plasma.

The increasing amount of research confirming the developmental toxicity of PFAAs in animal studies, coupled with their detection in human cord blood and milk, supports the need for examining the disposition of PFAAs during pregnancy/lactation in an appropriate animal model in order to fully establish the association between prenatal/neonatal exposure and offspring effects. While other studies have examined the pharmacokinetics of PFAAs in limited contexts, little data currently exist on the disposition of PFOA in pregnant and lactating mice or their offspring. We recently developed an analytical method for the analysis of PFOA in mouse serum, urine, milk, mammary tissue, amniotic fluid, and pups [24]. Utilizing these methods, we report here data on the distribution of PFOA in various matrices of pregnant and lactating CD-1 mice, as well as the serum concentration and total body load of their offspring, following a single exposure of PFOA on GD 17. These data will allow us to reduce the uncertainties in risk assessment for this particular PFAA.

2. Materials and methods

2.1 Chemicals

PFOA (ammonium salt; >98% pure), used in animal exposures, was purchased from Fluka Chemical (Steinheim, Switzerland). PFOA was completely dissolved by agitation in deionized tap water, in which PFOA was below the level of detection (LOD – 0.5 ng/L for water), and prepared fresh just prior to use.

2.2 Animals

All animal studies were conducted as approved by the National Health and Environmental Effects Research Laboratory Institutional Animal Care and Use Committee. Confirmed timed pregnant female CD-1 mice (n=100) were purchased from Charles River Laboratories (Raleigh, NC). Pregnant mice were received at the U.S. EPA's Laboratory Animal Care facility on gestation day (GD) 14 (day of sperm-positive designated as GD0). Upon arrival, mice (approximately 12 weeks old) were weighed and randomly distributed among PFOA treatment groups. They were housed individually in polypropylene cages with Alpha-dri (Shepherd Specialty Papers, Kalamazoo, MI) bedding and nesting materials. They were provided pelleted chow (LabDiet 5001, PMI Nutrition International LLC, Brentwood, MO) and tap water *ad libitum* (both contained PFOA at concentrations below the LOD). Animal facilities were controlled for temperature (20-24°C) and relative humidity (40-60%), and kept under a 14:10-h light-dark cycle. Mice (n=25/dose group) received either water vehicle or a single dose (0.1, 1.0 or 5.0 mg/kg) of PFOA (in water; 10 μl/g) by oral gavage on GD17.

2.3 Animal Assessments and Sample Collection

Live dam body weights were recorded on GD17, GD18 (prior to parturition), PND1 (day after parturition), and PNDs 2, 4, 8, 11, and 18. On GD18, 24 hr after the PFOA exposure, five dams in each dose group were sacrificed and trunk blood, urine, amniotic fluid (fluid immediately surrounding each fetus), and the 4th and 5th mammary gland were collected. Liver weight, total number of fetuses (live, dead, or resorbed), and fetal weights were determined. One entire fetus from each litter was euthanized by decapitation and quick frozen on dry ice in a 15 ml screwcap vial. Remaining fetuses were quickly euthanized and discarded. The dam mammary gland, urine, and amniotic fluid were kept on ice, and then frozen until assayed. The trunk blood was allowed to clot; serum was collected after centrifugation and frozen until assayed. All samples were kept frozen at -80° C.

A similar routine was followed on PND1 (48 hr after exposure, n=5 dams/dose group). Weights of the dam, pups, dam liver, and the number of live pups in each litter were recorded. A single pup from each litter was weighed, euthanized, and quick frozen in a collection vial (including all blood). Blood from all remaining pups in each litter was pooled into a single vial, allowed to clot, and separated to serum by centrifugation. Dam and pup serum, dam urine and mammary tissue were frozen until assayed. All remaining litters, in all dose groups, were equalized to 10 pups each on PND1. Biological samples, including a single pup and pup serum, as described for PND1, were also collected on PNDs 4, 8 and 18 (n=5 dams/dose group), at the same time of day.

Milk collection was attempted, following administration of oxytocin (1U/ml, i.p., 20 min prior to milking) on both GD 18 and PND 1, but was unsuccessful. Milk was

collected on PNDs 2, 8, 11, and 18 following a 2 hr separation of the pups from the dam and an oxytocin stimulus. The milk was vacuum aspirated using low, pulsatile pressure, into a pre-weighed microcentrifuge tube. Collected milk was weighed and frozen until analyzed. Biological samples including urine, dam and pup serum, amniotic fluid, mammary tissue, whole pup, and milk were analyzed for PFOA using the methods described briefly below and in our companion paper [24].

2.4 PFOA sample analyses

Briefly, the analysis of PFOA was performed using a Waters Acquity™ Ultra Performance liquid chromatography system interfaced with a Waters Quattro Premier XE triple quadrupole mass spectrometer (UPLC-MS/MS) (Waters, Milford, MA). Either 25 or 50 μL of serum and amniotic fluid (50 μL used for controls), 20 μL aliquots of urine and milk, and 300 μL of pup or mammary tissue homogenates were utilized as starting material for these analyses. Samples were extracted, purified, and concentrated or diluted exactly as described by Reiner et al. [24]. 10-40 μL of the prepared sample, depending on the concentration of the original exposure, was injected and run on the UPLC-MS/MS [24]. Refer to Reiner et al. [24] for method performance and quality control steps that were performed to insure the precision and accuracy of the methods used. The limit of quantitation (LOQ) for these experiments were 5 ng/ml (serum), 1 ng/ml (amniotic fluid, urine, milk), and 1 ng/g (whole pups, mammary tissue).

2.5 Urinary creatinine measures

Creatinine concentrations were measured as a basis to evaluate PFOA in mouse urine. The QuantiChrom creatinine assay (BioAssay Systems, Hayward, CA) exhibited an LOD of 0.10 ng/ml and was linear up to 300 ng/ml. Thirty µl of each urine sample was prepared and evaluated at 510 nm singly or in duplicates (five duplicates per set of 20 samples) according to the manufacturer's instructions. The inter-assay coefficient of variation (CV) ranged from 4.0–6.8% and the intra-assay CV ranged from 0.3-16.1%, with an average of 4.9%. The assay standard accuracy ranged from 0.2-8.4%. Urinary PFOA is reported as corrected for creatinine concentrations (ng PFOA/g creatinine).

2.6 Computations and Statistics

Reported PFOA concentrations have been adjusted for dilution or concentration factors, as well as creatinine levels (ng/g; urine), or the weight of the tissue evaluated (ng/g; mammary tissue and whole pups). Serum, amniotic fluid, milk and urinary concentrations are reported as ng/ml. Averages, proportions, and statistical comparisons were calculated with SAS 9.1 (SAS Institute; Cary, NC). Statistical significance was determined using a Proc GLM ANOVA, with a Dunnett's post-hoc comparison, and significance was set at $p<0.05$.

3. Results

3.1 Biological Outcomes

This is the first study to report single dose disposition of PFOA in pregnant and lactating mice and their offspring. The doses chosen were based on previous reports in CD-1 mice [3, 14, 16] demonstrating developmental health outcomes following multiple gestational PFOA exposures. A single PFOA exposure on GD17 did not affect the number of live fetuses (on GD18), implantation sites, or live-born pups (on PND1), or dam body weights (data not shown). Unlike studies using multiple gestational PFOA exposures [13, 25], there was no change in pup body weight, dam liver weight, and dam liver:BW ratios, within the PFOA dose range administered in this study (Figure 1). The rise in dam liver:BW ratio between GD18 and PND1, which persisted until weaning, was due to the dramatic decrease in body weight at parturition, as this single late gestation PFOA exposure failed to change mean liver weight in exposed dams, compared to control values, at any time evaluated.

3.2 PFOA Concentrations Prior to Birth

The mean concentration of PFOA in the amniotic fluid and serum of the exposed dams 24 hr after exposure is shown (Figure 2; amniotic fluid controls average 3.8 ng/ml). The average concentration of PFOA detected in dam serum was about twice the amniotic fluid concentration at each dose evaluated (amniotic fluid was 68.8, 51.8, and 40% of dam serum levels at 0.1, 1, and 5 mg PFOA/kg BW, respectively). A comparison of the amount of PFOA in an entire GD18 fetus (body burden/pup±standard error of the mean [SEM]; Figure 5) to the GD18 PFOA concentration in amniotic fluid (ng/ml; assuming 1

ml total volume) reveals 2.3-, 3.1-, and 2.7-fold increased PFOA in the pup vs. the fluid in which it was contained *in utero* for 0.1, 1, and 5 mg/kg dose groups, respectively.

3.3 PFOA Concentrations in the Dams

The serum and urine PFOA concentrations were evaluated in dams that were nursing litters of approximately 10 pups (PND1 equalized; minimal pup loss over time). As expected, dam sera contained the highest PFOA concentrations of any matrix evaluated, regardless of dose (Figure 3; all serum controls <LOQ). The rise in circulating serum PFOA with increasing dam exposures was proportional to the change in dose delivered, regardless of stage of lactation (i.e., mean 9.9-fold and 5.1-fold increases between 0.1-1.0 mg/kg and 1.0-5.0 mg/kg exposures, respectively).

A one-time PFOA exposure of 0.1 mg/kg produced an average dam serum PFOA concentration (Figure 3A) of 144-226 ng/ml at 24 and 48 hr after exposure, respectively, which was reduced to an average of 44 ng/ml near the peak of lactation (PND8), and had risen to a mean of 123 ng/ml by PND18, a time when the pups' primary caloric intake came from rodent chow and not milk. The U-shaped serum concentration curve observed in the 0.1 mg PFOA/kg dose group was also present in the 1 and 5 mg/kg exposure groups.

As shown in Figure 3 (A-C; control urine and mammary gland PFOA <LOQ), although the concentrations of PFOA cannot be compared directly between serum, urine, and mammary tissue, due to the difference in units, it was evident that much less PFOA was being excreted in dam urine than was present in dam serum, and that mammary tissue contained a considerable amount of PFOA. While a U-shaped response in dam

excretion of PFOA (urine) was not as pronounced as that of serum, mammary tissue demonstrated a strong U-shaped response, with the lowest concentrations measured near the peak of lactation, and a significant rise in concentration apparent again at PND18 ($p<0.05$).

When aspirated milk PFOA values were evaluated (Figure 3D; 1 control >LOQ), a U-shaped curve over time was again evident. As depicted in Table 1, the percentage of PFOA in milk (compared to serum) was substantial. Comparing the milk concentrations to the closest matched dam serum concentrations (by time), the amount of PFOA in the milk consistently ranged from 1/10 to 1/2 that of dam serum PFOA across dose and time. It appeared that the day of lactation on which milk PFOA was measured had an important influence on this relationship. The milk:serum PFOA ratio was greatest in early and late lactation (PND2 and PND18), ranging from 15-56% (means of 33% early and 26% late), while near the peak of lactation (PND8 and 11), the PFOA milk:serum ratio ranged from 11-27% (mean 17.7%). It was not possible to accurately measure the volume of milk obtained at aspiration, but precise weights were compared. On PNDs 2, 8, 11, and 18, the average weight of milk obtained via aspiration of control mice was 0.072, 0.1906, 0.2547, and 0.0457 g, respectively, demonstrating over a 3.5-fold increase in weight from PND2 to 11 and a 5.6-fold drop from PND11 to 18.

3.4 PFOA Concentrations in the Pups

Pup serum PFOA concentration was evaluated on PNDs 1, 4, 8, and 18. In comparing the average PFOA concentrations in PND1 pups vs. their respective dams (Figure 4A; whole control pups and control serum < LOQ), it appeared that circulating

pup serum PFOA concentrations were significantly higher than those measured in dams, regardless of dose ($p<0.05$). Although pups possessed a substantially higher serum PFOA concentration than dams, the difference in pup and dam blood volumes at those stages of pup development are considerable. Regardless of those differences, heightened circulating PFOA in pup sera reflected increased exposures, proportional to dose throughout lactation (i.e., mean 10.4-fold and 4.3-fold increases between 0.1-1.0 mg/kg and 1.0-5.0 mg/kg exposures, respectively).

Unlike their dams, pups did not demonstrate U-shaped serum PFOA concentration curves (Figure 4B). Pup serum PFOA concentrations continued to exceed the average dam serum PFOA concentrations over time, until PND18 when the pup and dam concentrations approached 1:1. When the PFOA concentration (ng/g) was evaluated in whole pups (pup and blood; Figure 5 left panels), a decline in PFOA concentration was detected over time, across all doses. However, when the rapidly increasing body weight of the pups was taken into consideration to calculate the total amount of PFOA in the neonate (as shown in Figure 1), a completely different trend was noted (Figure 5 right panels). Regardless of exposure dose, PFOA body burden (adjusted for weight) rose through the peak of lactation and had begun to decline by PND18, demonstrating an inverse U-shaped curve. When the administered PFOA dose and measured body burden in whole pups (body weight taken into effect) were compared the administered PFOA:measured PFOA ratio was no longer proportional throughout lactation, and unlike the ratios reported for dam and pup serum PFOA. Mean body burden ratios of 13.2-fold (range 11.1-17.8) and 4.3-fold (range 3.2-5.1) increases between 0.1-1.0 mg/kg and 1.0-5.0 mg/kg exposures, respectively, were determined.

4. Discussion

These data confirm that on a concentration-based comparison, gestationally PFOA-exposed pups exhibited a significantly larger serum PFOA load than their dam. That substantial serum PFOA load in pups was evident 24 hr after a single exposure, and was apparently due to blood-borne (transplacental) transfer. Another important discovery is the U-shaped PFOA concentration over time, regardless of dose, in the dam mammary tissue, milk, and serum. This unique PFOA response was not detected in pups or pup serum, and was evident to a lesser extent in the dams' urinary excretion curves. However, when PFOA body burden in whole pups was the unit of measure, an inverse U-shaped curve was apparent, and the PFOA burden of pups is proposed to increase due to milk-borne PFOA intake.

The decline in concentration seen in the milk, mammary and serum U-shaped curves is hypothesized to be due to hydro-dilution associated with increased blood and milk volumes. Several physiological conditions are changed during lactation that have been well documented in rats and directly relate to mice as their lactation period is of the same length. A decrease in total plasma proteins due to increased blood volume, cardiac output, and blood flow to certain tissues, such as the mammary gland has been reported in rats [26, 27]. Elevated blood volume is due to increased plasma volume [27]. Milk yield (g/hr) in rats was reported to reach its peak by PND 10 [27] and the rat mammary gland reaches its maximum size (as % body weight) by PND15 [26], with a steep rise in size from PND5-15. Rat mammary gland blood flow and volume of milk produced are directly related, when measured on PND15 [26]. Total serum proteins are lower in lactating rats that those measured in non-lactating rats [27], and in humans, serum

albumin concentration decreases during pregnancy and early lactation [28]. Further, at 14 d postpartum, the cardiac output of lactating rabbits was 30% higher than that in non-lactating animals, and the mammary gland was the only organ shown to increase in weight, relative to body weight [29].

Although a complete set of data that could address the exact reason for the U-shaped curves during lactation was not collected in this study, the aspirated milk weights did reveal a dramatic increase in milk volume (assumed due to weight change) from PND2 up to the peak of lactation (PND11). This dramatic change in volume (weight) may explain the decrease in milk PFOA concentration seen between PND2 and PND11. PFOA also appears to concentrate in serum and milk near the end of lactation (PND18, for example) when pups are eating more chow and suckle less often. Mammary gland blood flow has been reported to decrease by half in a 24 hr period, when suckling rat offspring are removed from the dam [26], and this fall in mammary blood flow is directly associated with decreased cardiac output and % blood flow used by the mammary gland. In this study a precipitous drop in weight of milk collected between the peak of lactation and PND18 was noted, indicating a rapid decrease in milk volume. Therefore, the U-shape of the dam PFOA curves are proposed to be driven by physiological dilution and concentration of the PFOA load over the period of lactation, reaching the greatest dilution at or near the peak of lactation when the milk volume produced by the dam and consumed by the pups is the greatest. Increased consumption of milk up to PND11 likely directly contributed to the accumulation of body burden in the pup over this life stage.

A significant contribution of milk-borne PFOA transfer in CD-1 mice was detected in these studies. Previous reports in rats [22] and humans [18] have estimated

that the dam PFOA milk:serum distribution ratio was 0.1 and 0.01, respectively. In the present study, the distribution ratio ranged from slightly more than 0.5 to 0.1 in mice, depending on dose, with the lowest doses tested demonstrating the highest ratios over time. If the milk PFOA concentrations had been measured near the peak of lactation only (days 8-11), the 0.1 milk:sera distribution estimate previously reported for rats in mid-lactation [22] may have also been presumed true in mice. However, at two periods during lactation (early and late) spikes of increased milk:serum ratios appeared, regardless of dose, with a substantial peak in milk PFOA concentrations on PND2. Although volumes of milk large enough to perform analytical measures prior to PND2 were not able to be obtained, we suspect, based on the significant PFOA concentrations in the PND1 mammary gland, that substantial milk PFOA concentrations would have been evident on PND1, as well, primarily due to being condensed in small milk volumes.

In previous reports by Lau [13], Wolf [16], White [14] and co-workers, decreased body weight gain and neonatal mortality were evident on several days just after birth in CD-1 mouse litters gestationally exposed to 3 mg/kg PFOA and higher. In fact, in a cross-foster study [16] demonstrating decreased body weight gain at 5 mg/kg from *in utero* exposure only, significant decreases in body weight gain were detected in the 3 mg/kg dose group only when *in utero* exposed mice were also allowed to nurse from a PFOA exposed dam. Even at 5 mg/kg, there was no evidence of decreased pup body weight or neonatal mortality in the current study, following a single gestational PFOA exposure. Our PFOA measurements in whole pups indicate that the PFOA body burden accumulates in early life, and begins a decline as pups mature, open their eyes, and begin to eat chow and drink water. Our data and those demonstrating deleterious health

outcomes suggest that the milk of gestationally PFOA-exposed mice was a major source of continued exposure to this compound for the developing pups.

As expected, large differences in dam and pup serum PFOA concentrations from those previously reported [14, 16] were noticed, and those differences bring to light the issue of single vs. multiple dose kinetics. As noted for PFOS, single dose kinetics may differ substantially from those involving repeated doses [30]. Concentration dependent changes in clearance can result in discrepancies between single and repeated dose kinetics.

A limited number of epidemiological studies have revealed associations between health outcomes (birth weight, head circumference) and cord blood or maternal serum PFOA concentrations in humans [20, 21], while other studies failed to detect associations with later developmental milestones in infants [31]. Several studies have now measured PFAAs in human milk [17-19, 32, 33], however only one study has been able to approximate the milk:serum relationship of PFOA transfer [18]. The reported 0.01 ($1/100^{th}$) relationship was determined from a single voluntarily contributed sample at 3 weeks postpartum. According to the mouse milk:serum PFOA distribution over time that we report herein, the values reported in one human [18] and rats [22] may not be representative of the PFOA distribution to milk throughout lactation in those species.

In conclusion, these studies confirmed and further defined considerable PFOA exposures to mouse offspring following a single gestational exposure. They also demonstrated the accumulation of chemical over time in whole pups, which likely results from milk-borne PFOA, an exposure that had previously been incompletely assessed in other species. A single 0.1 mg/kg PFOA exposure to a pregnant mouse induced

circulating serum PFOA concentrations of 44-216 ng/ml in dams and 117-326 ng/ml in pups; values similar to or lower than serum PFOA concentrations of children that were accidentally exposed via DuPont production plant emission [34]. Because of evidence [15, 35] demonstrating neonatal and latent health effects following developmental exposures to PFOA in mice, associated with higher circulating PFOA levels than those reported here, continued studies evaluating exposure-effect relationships are warranted in children.

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Figure legends:

Figure 1. Dam tissue weights and average pup weights following a single gavage PFOA exposure on GD17. PFOA was without effect on several biological end points ($p>0.05$), such as dam body weight measured on several postnatal days (PND) and on gestation day (GD)18 (not shown). (A) Dam liver weight, (B) liver:body weight ratio, and (C) pup body weight over time or numbers of live pups or fetuses (not shown) were also unchanged by a single PFOA exposure. Data are shown as Mean ± SEM or as a mean ratio.

Figure 2. Comparison of gestation day (GD)18 dam serum and amniotic fluid PFOA concentrations. PFOA concentrations were significantly higher in dam serum than amniotic fluid at all doses evaluated ($p<0.05$). Data are shown as Mean ± SEM.

Figure 3. PFOA concentrations in exposed dams. PFOA concentrations were measured in dam serum (A; ng/ml), urine (B; ng/g creatinine), and mammary tissue (C; ng/g tissue weight) on postnatal days (PND) 1, 4, 8 and 18. PFOA concentrations were measured in aspirated milk samples collected on PNDs 2, 8, 11, and 18 (D; ng/ml). Although panels A-C and B-D cannot be directly compared (due to different units), the U-shaped concentration curve present in dam serum (regardless of dose) was also detected in mammary tissue and aspirated milk. Data are shown as Mean ± SEM. †Denotes a single reliable measurement at this time due to insufficient volumes in other dams at this dose and time.

Figure 4. Neonatal transfer of PFOA to pups. (A) A significantly higher PFOA concentration in pup vs. dam serum on PND1 was noted ($p<0.05$; v:v). (B) Pooled pup serum PFOA concentrations did not demonstrate a U-shaped curve, but gradually

declined over time, presumably due to dilution of dose by increased growth-related blood volume. Data are shown as Mean ± SEM.

Figure 5. Whole pup PFOA concentrations. PFOA concentrations were measured in a representative whole pup (pup and blood; ng/g; left panels) from each litter. Although there is a consistent downward trend in PFOA concentration over time, the rapidly increasing blood volume and body weight changes must be taken into consideration when interpreting these data. Body weight-adjusted values (right panels; [ng/g PFOA measures*g body weight = body burden]) demonstrate an accumulation of exposure until late in the lactational period. Data are shown as Mean ± SEM.

Figure 1.

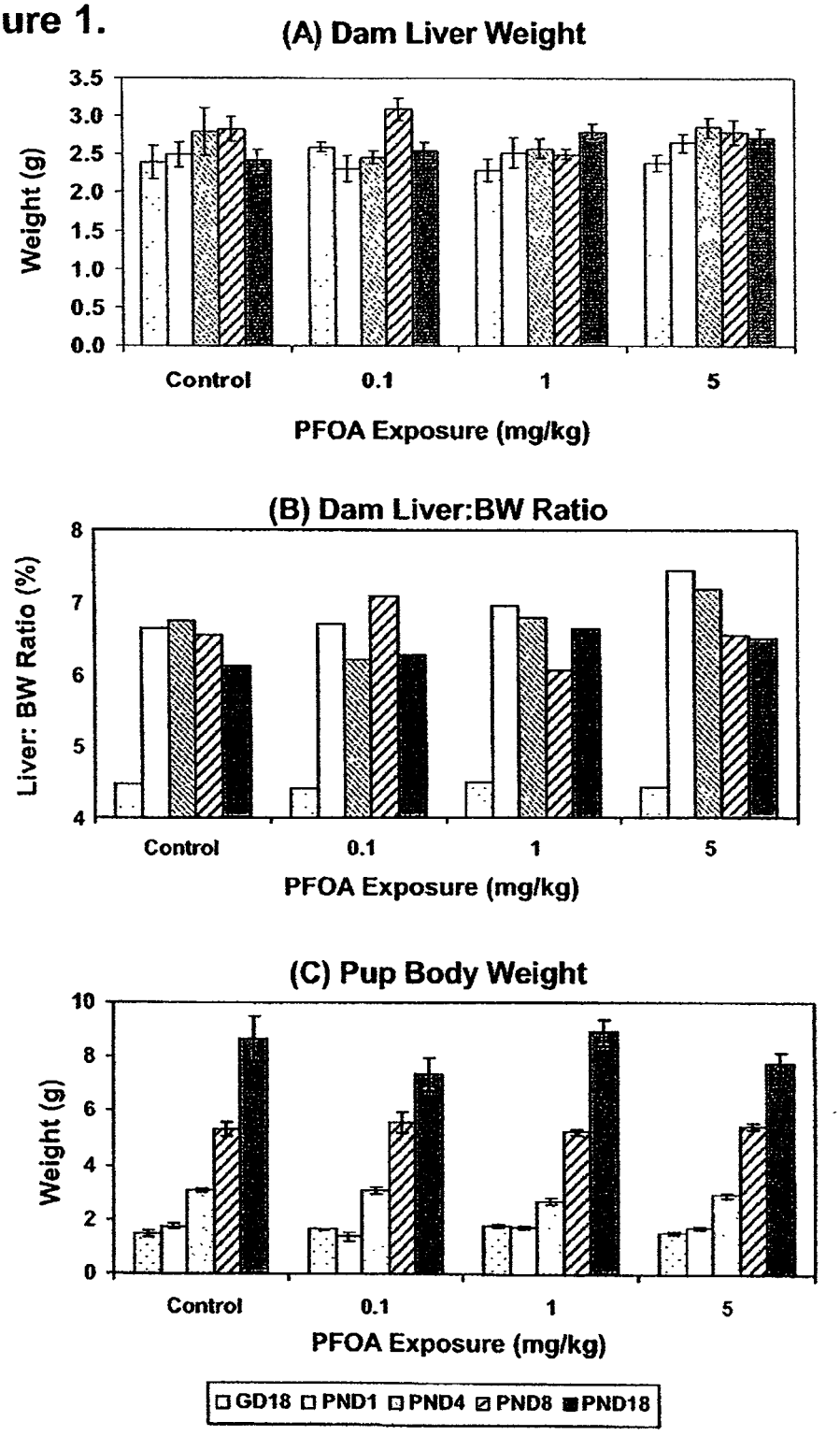
(A) Dam Liver Weight
Weight (g)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
0.1
1
5
PFOA Exposure (mg/kg)
(B) Dam Liver:BW Ratio
Liver: BW Ratio (%)
8
7
6
5
4
Control
0.1
1
5
PFOA Exposure (mg/kg)
(C) Pup Body Weight
Weight (g)
10
8
6
4
2
0
Control
0.1
1
5
PFOA Exposure (mg/kg)
GD18 PND1 PND4 PND8 PND18

Figure 2.

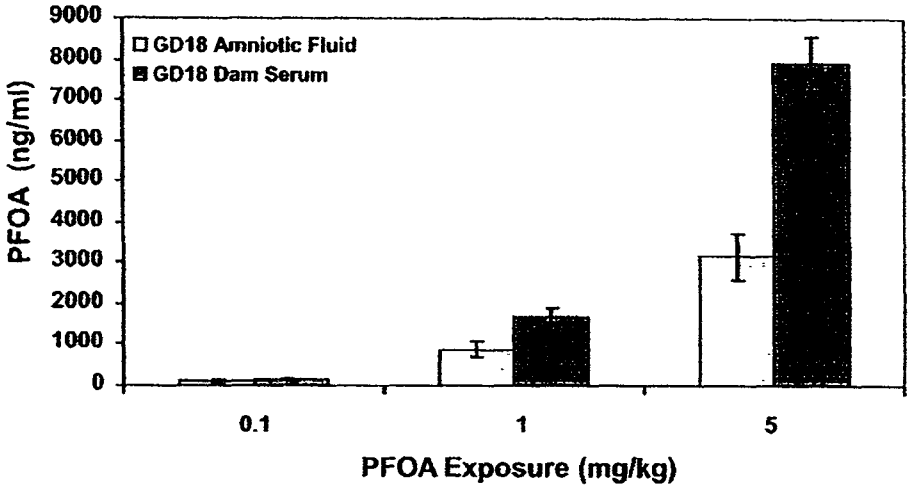
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
PFOA (ng/ml)
GD18 Amniotic Fluid
GD18 Dam Serum
0.1
1
5
PFOA Exposure (mg/kg)

Figure 3.

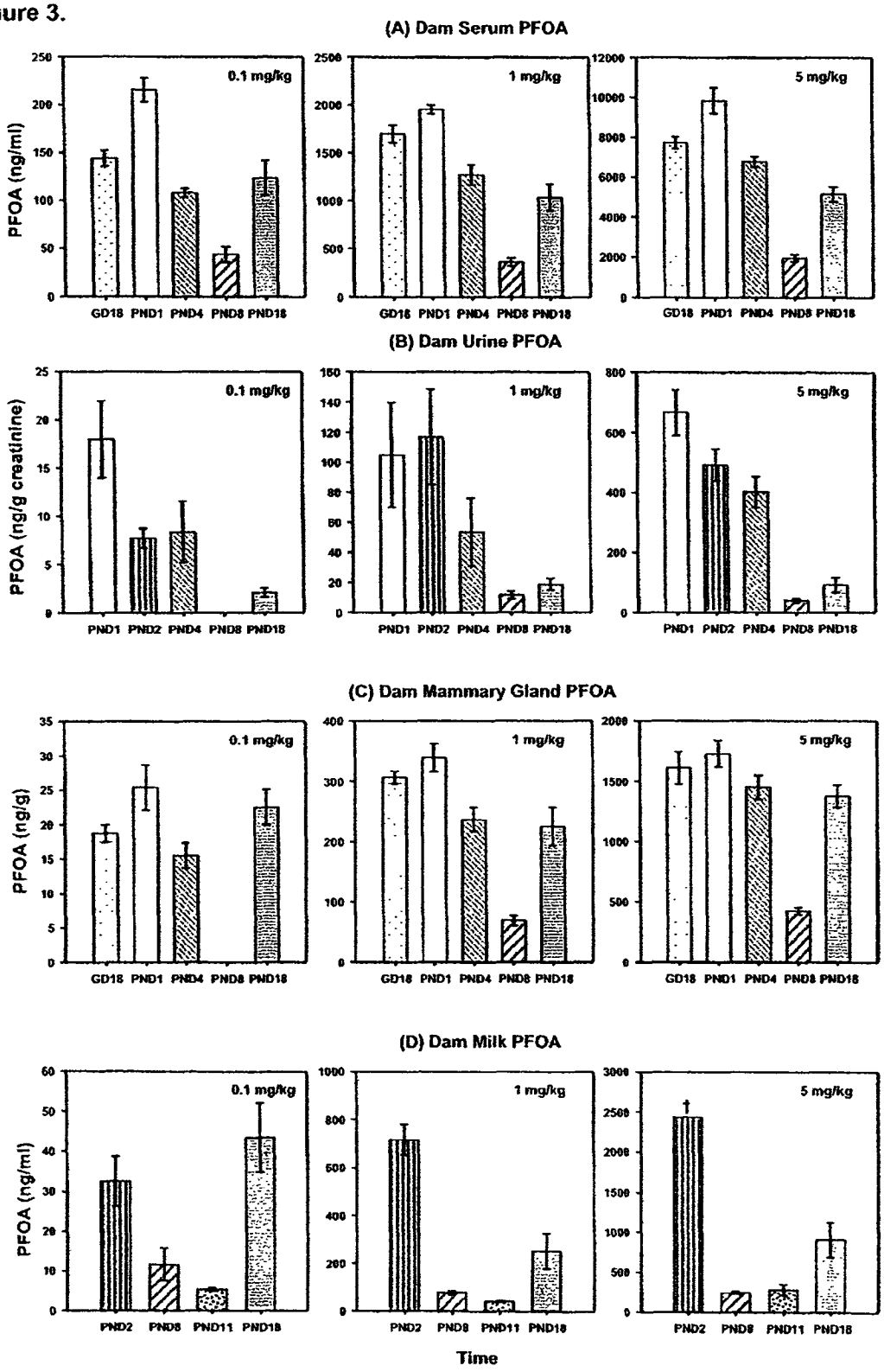
(A) Dam Serum PFOA
PFOA (ng/ml)
0.1 mg/kg
1 mg/kg
5 mg/kg
GD18 PND1 PND4 PND8 PND18
(B) Dam Urine PFOA
PFOA (ng/g creatinine)
PND1 PND2 PND4 PND8 PND18
(C) Dam Mammary Gland PFOA
PFOA (ng/g)
(D) Dam Milk PFOA
PFOA (ng/ml)
PND2 PND8 PND11 PND18
Time

Figure 4.

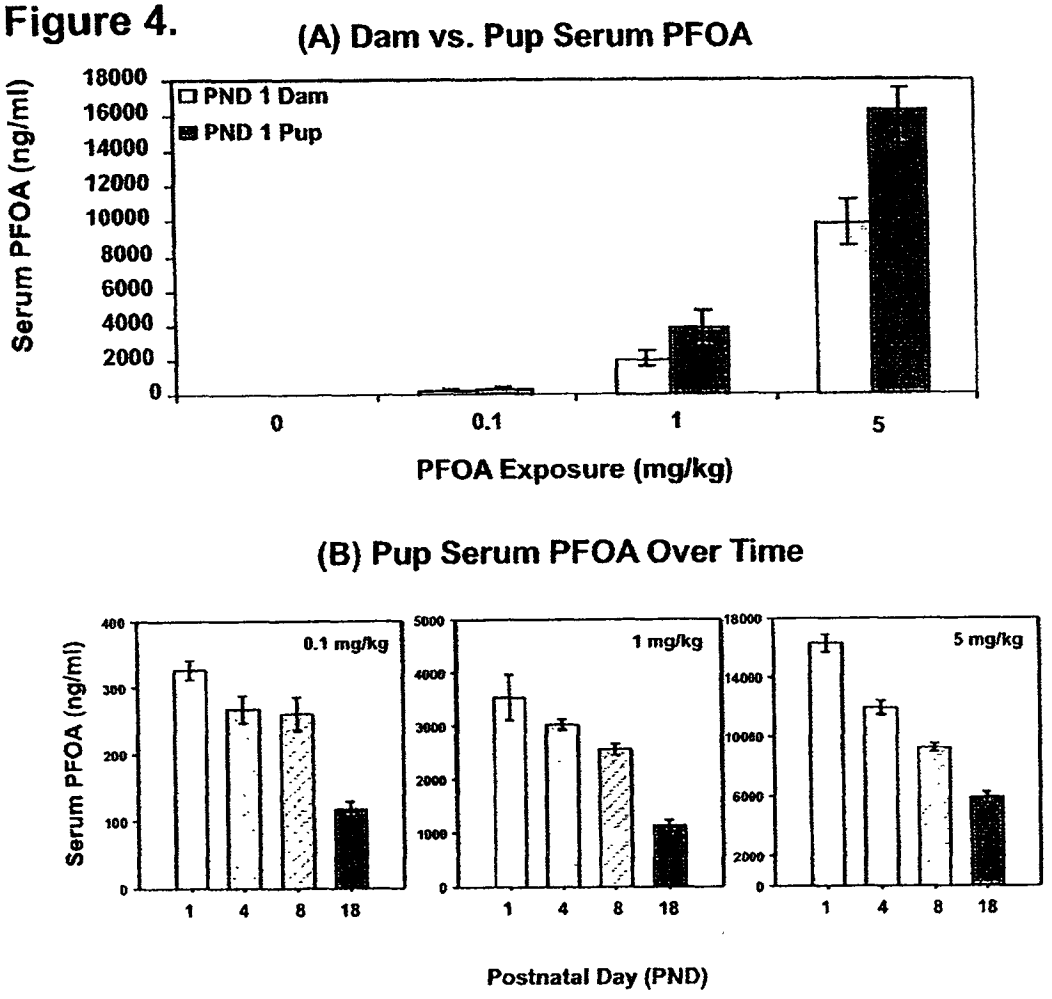
(A) Dam vs. Pup Serum PFOA
Serum PFOA (ng/ml)
PND 1 Dam
PND 1 Pup
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
0
0.1
1
5
PFOA Exposure (mg/kg)
(B) Pup Serum PFOA Over Time
Serum PFOA (ng/ml)
0.1 mg/kg
1 mg/kg
5 mg/kg
1
4
8
18
Postnatal Day (PND)

Figure 5.

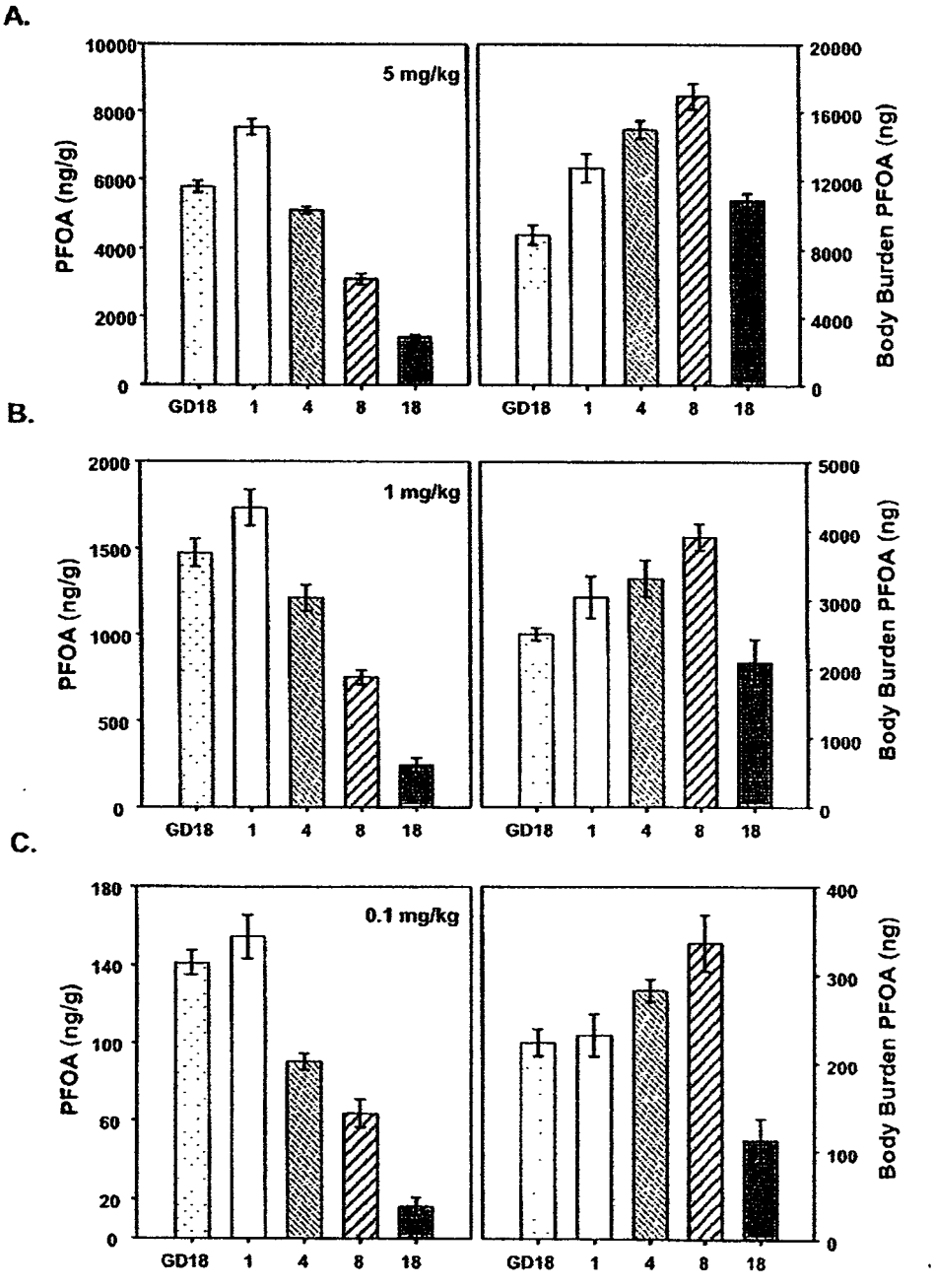

Table 1. Milk-borne PFOA† as a percentage of dam serum concentrations over lactation.

Single GD17 PFOA exposure	PND 1 serum PFOA comparison	PND 4 serum PFOA comparison	PND 8 serum PFOA comparison	PND 8 serum PFOA comparison	PND 18 serum PFOA comparison
	PND 2 milk		*PND 8 milk*	*PND 11 milk*	*PND 18 milk*
0.1 mg PFOA/kg	15%	31%	27%	11%	36%
1.0 mg PFOA/kg	37%	56%	21%	21%	24%
5.0 mg PFOA/kg	25%	36%	13%	13%	18%

† PFOA= perfluorooctanoic acid, GD=gestational day, PND=postnatal day. The milk:serum PFOA ratio reported above was calculated as: [concentration of milk PFOA/concentration of serum PFOA]*100=% milk:serum for each dam within a dose group. These values were averaged and reported above.

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Polyfluoroalkyl chemicals in the serum and milk of breastfeeding women

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ABSTRACT

Polyfluoroalkyl chemicals (PFCs) comprise a group of man-made organic compounds, some of which are persistent contaminants with developmental toxicity shown in laboratory animals. There is a paucity of human perinatal exposure data. The US EPA conducted a pilot study (Methods Advancement in Milk Analysis) including 34 breastfeeding women in North Carolina. Milk and serum samples were collected at 2–7 weeks and 3–4 months postpartum; 9 PFCs were assessed in milk and 7 in serum. Perfluorooctane sulfonic acid (PFOS), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), and perfluorohexane sulfonic acid (PFHxS) were found in nearly 100% of the serum samples. PFOS and PFOA were found at the highest concentrations. PFCs were below the limit of detection in most milk samples. Serum concentrations of PFOS, PFOA and PFHxS were lower ($p < 0.01$) at the second visit compared to the first visit. Living in North Carolina 10 years or longer was related to elevated PFOS, PFOA and PFNA ($p \leq 0.03$). These pilot data support the need to further explore perinatal PFC exposures and potentially related health effects, as planned in the upcoming National Children's Study which provided the framework for this investigation.

1. Introduction

Polyfluoroalkyl chemicals (PFCs) comprise a large group of man-made fluorinated organic compounds used in numerous consumer products and industrial applications such as food packaging material, non-stick cookware, protective coatings for textiles, carpets, and paper, surface car coatings or treatments, as well as in surfactants for commercial and industrial applications [1]. PFCs, and more specifically perfluoroalkyl acids (PFAAs), have been detected in wildlife, fish used for human consumption, and sera of humans in many different geographical areas worldwide [2–19]. Nationally representative US sera biomonitoring data in subjects 12 years and older demonstrated widespread exposure to perfluorooctane sulfonic acid (PFOS), perfluorooctanoic acid (PFOA), and perfluorononanoic acid (PFNA) during the last decade [20,21].

Exposures of lactating women and young children to PFCs have not been frequently studied, although a number of animal and recent human studies have suggested transfer to breast milk and across the placental barrier [22–26]. Developmental and reproductive health effects in animals, including reduced birth weight and gestational length, developmental delays and structural defects especially in relation to PFOA and PFOS exposure have increasingly raised concerns, although the developmental toxicity in laboratory animals was shown at doses 100–500 times of those seen in human sera [2,27–29]. Some exposure assessments in cord blood suggested that PFAAs can also cross the placental barrier in humans [30,31]. Apelberg et al. [23] recently reported average cord blood concentrations of 4.9 ng/ml (PFOS) and 1.6 ng/ml (PFOA) ($n = 299$), while Spliethoff et al., reported the detection of PFAAs in new born blood spots confirming the transfer of PFAAs *in utero* [32].

In two recent epidemiological studies, PFAA cord blood concentrations were related to anthropometric indicators of fetal growth at birth, and maternal pregnancy serum PFAA concentrations were associated with child birth weight [22,24]. Based on the Danish National Birth Cohort, inverse associations were reported between gestational PFOA exposure and birth weight while no effects were reported for markers of fetal growth at birth, or postnatal developmental milestones [24,33].

Abbreviations: CI, confidence interval; IQR, interquartile range; LOD, limit of detection; LOQ, limit of quantification; Pfaas, perfluoroalkyl acids; PFOSA, perfluorooctane sulfonamide; Et-PFOSA-AcOH, 2-(N-ethyl-perfluorooctane sulfonamido) acetic acid; Me-PFOSA-AcOH, 2-(N-methyl-perfluorooctane sulfonamido) acetic acid; PFHxS, perfluorohexane sulfonic acid; PFOS, perfluorooctane sulfonic acid; PFOA, perfluorooctanoic acid; PFNA, perfluorononanoic acid; PFC, polyfluoroalkyl chemicals; WTC, World Trade Center.

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Data on human milk PFC concentrations are still sparse. The available data based on small sample sizes from China [34], Sweden [35], Germany and Hungary [36], suggested detectable levels of predominantly PFOA and PFOS. The concentrations of PFOS (131 pg/ml) and PFOA (43.8 pg/ml) in 45 milk samples collected in 2004 from women aged 22–43 years residing in Massachusetts have been reported recently [25]. Studies investigating the partition of PFCs into milk are largely lacking. One earlier study in Sweden ($n = 12$) suggested transfer of only about 1% of PFC concentration in serum into milk [35]. Temporal concentration changes in serum or milk of lactating women are unknown, as no study has assessed concentrations in the same woman at two time points during lactation.

To evaluate infant and maternal exposure to PFCs and to a range of other environmental components, as well as to compare concentrations across biological fluids [37], the US Environmental Protection Agency (US EPA) conducted a pilot study entitled Methods Advancement for Milk Analysis (MAMA). This pilot study was carried out to develop reliable collection and analysis methods for the National Children's Study, including 100,000 children from pre-conception to age 21 [38]. We previously reported the MAMA findings regarding phthalates [37] and the biological components of human milk [39].

2. Materials and methods

2.1. Study design and population

The design of the EPA MAMA study and basic methods has been described in detail previously [39]. In brief, 34 healthy, English-speaking breastfeeding women between 18 and 38 years of age were recruited via newspaper advertisements, university email publications, and fliers distributed to clinicians specializing in women's health or pediatrics by an EPA contractor (Westat Inc., Chapel Hill, NC). The questionnaire assessment and the collection of milk and serum specimens were conducted at the EPA's Human Studies Facility clinic (Chapel Hill, NC) between December 2004 and July 2005. Women were breastfeeding their first, second or third child and were not required to exclusively breastfeed for participation in this study. The women donated milk and serum samples at 2–7 weeks (1st visit: $n = 18$ milk; $n = 34$ serum) and at 3–4 months (2nd visit: $n = 20$ milk; $n = 30$ serum) postpartum. The participation of human subjects in the MAMA study was approved by the Institutional Review Boards of the University of North Carolina's School of Medicine (IRB number 03-EPA-207) and the Centers for Disease Control and Prevention (IRB number 3961). The women participated in verbal and written informed consent prior to administration of a comprehensive questionnaire which did not include questions regarding the offspring of study participants [39].

2.2. Questionnaire

A questionnaire regarding maternal residence, occupation, and dietary and lifestyle factors was administered to participants at the first clinic visit. Questionnaire items were selected to address potential routes of exposure to multiple environmental chemicals (phthalates, phenols, PCBs, dioxins, PFCs, persistent organic pollutants, metals, and brominated flame retardants). The current analysis included the following questions that were thought to potentially relate to PFC exposure routes: "How long have you lived in North Carolina?" [40–42] and "Does your home have an enclosed garage attached?". The latter question was selected as some applications used in and around cars contain PFCs, e.g., external and internal surface car coatings or treatments.

2.3. Sample collection and preparation

The women were asked to fast for 1.5 h before sample collection. The MAMA sample collection procedures for serum and milk were published previously [39]. Sampling details, including time of day (between 9 AM and 2 PM) and the amount of bodily fluid collected, were recorded in the collection log. Milk (90 ml or ~3 oz) was expressed in the EPA clinic using a commercially available electric breast pump (Medela, McHenry, IL). Milk was pumped into PFC-free bottles and divided into 3 ml aliquots in PFC-free polypropylene-tubes. Women's blood samples (about 20 ml), were collected into non-heparinized glass vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) by an EPA nurse via venipuncture. After 1 h at room temperature to allow for clotting, blood samples were spun at 3000 rpm for 15 min at room temperature and serum was collected. All samples were stored at −20 °C and shipped on dry ice to the CDC's Division of Laboratory Sciences, National Center for Environmental Health (Atlanta, GA) for analysis. At the CDC, all samples were stored at or below −20 °C until analyzed.

Table 1
Limits of quantification (LOQ) in milk and limits of detection (LOD) in serum (ng/ml).

Polyfluoroalkyl chemicals	Milk LOQ	Serum LOD
2-(N-ethyl-perfluorooctane sulfonamido) acetic acid	0.60	0.20
2-(N-methyl-perfluorooctane sulfonamido) acetic acid	0.60	0.20
Perfluorobutane sulfonate	0.30	[a]
Perfluorodecanoate	0.60	[a]
Perfluorohexane sulfonate (PFHxS)	0.30	0.10
Perfluorononanoate (PFNA)	0.30	0.10
Perfluorooctane sulfonamide (PFOSA)	0.15	0.05
Perfluorooctane sulfonate (PFOS)	0.60	0.05
Perfluorooctanoate (PFOA)	0.30	0.10

[a] Denotes not measured in serum.

2.4. Analysis of milk and serum for PFCs

In serum and milk, we determined the concentrations of PFOS, PFOA, PFNA, PFHxS, perfluorooctane sulfonamide (PFOSA), 2-(N-methyl-perfluorooctane sulfonamido) acetic acid (Me-PFOSA-AcOH), 2-(N-ethyl-perfluorooctane sulfonamido) acetic acid (Et-PFOSA-AcOH); perfluorobutane sulfonic acid and perfluorodecanoic acid were only measured in milk. The analytical method involved automated solid-phase extraction (SPE) coupled to reversed-phase high performance liquid chromatography (HPLC)–tandem mass spectrometry (MS/MS). Samples were run in singlets and were re-analyzed only if the water and/or matrix blanks were above 3 × limit of detection (LOD). The analytical procedures involving the use of standards, quality control, and blanks, as well as automated sample extraction were conducted as published previously [43–46]. The samples from both visits were analyzed together in March 2006 (serum) and November 2006 (milk).

For milk, sample preparation was conducted using automated off-line SPE [43]. One-ml of milk, to which we added 3 ml of 0.1 M formic acid and 50 μl of internal standard solution, was vortex-mixed and sonicated, and placed on a Zymark Rapid Trace Station (Zymark Corp., Hopkinton, MA). PFCs from the milk were extracted on an Oasis-HLB SPE column (Waters Corporation, Milford, MA). The SPE eluate was evaporated at 55 °C to ~100 μl under a stream of dry nitrogen (UHP grade) in a Zymark Turbovap evaporator, and reconstituted with 300 μl of 0.1% formic acid. The reconstituted milk extract (~400 μl) was transferred to a polypropylene autosampler vial for the on-line SPE-HPLC-MS/MS analysis, performed using a Surveyor HPLC system (ThermoFinnigan, San Jose, CA, USA), including one six-port switching valve (Rheodyne MX7960, Rohnert Park, CA, USA) and one additional Surveyor LC pump, coupled with a ThermoFinnigan TSQ Quantum Ultra triple-quadrupole mass spectrometer equipped with a heated electrospray ionization (HESI) interface. The HPLC pump operated at a 300 μl/min flow rate with 20 mM ammonium acetate (pH 4) in water (mobile phase A) and acetonitrile (mobile phase B). The extract was injected into the liquid chromatograph system for concentration of the PFCs by on-line SPE on a Betasil C8 precolumn (3 mm × 10 mm, 5 μm; ThermoHypersil-Keystone, Bellefonte, PA, USA), chromatographic separation on a Betasil C8 analytical HPLC column (2.1 mm × 50 mm, 5 μm; ThermoHypersil-Keystone), and detection and quantification by negative-ion HESI-MS/MS.

For serum, we used a modification of the on-line SPE coupled to HPLC-MS/MS approach described before [47]. Briefly, we added 250 μl of 0.1 M formic acid and 25 μl of internal standard solution to 100 μl of serum, and the spiked serum was vortex-mixed and sonicated. The samples were placed on a Symbiosis on-line SPE system (Spark Holland, Plainsboro, NJ) for the preconcentration of the analytes on a Polaris C18 cartridge (7 μm, 10 mm × 1 mm; Spark Holland). The analytes were transferred onto a Betasil C8 HPLC column (3 mm × 50 mm, 5 μm; ThermoHypersil-Keystone, Bellefonte, PA), separated by HPLC (mobile phase A: 20 mM ammonium acetate in water, pH 4; mobile phase B: methanol), and detected by negative-ion Turbolonspray-MS/MS on an API 4000 mass spectrometer (Applied Biosystems, Foster City, CA). Reportable breast milk PFC concentrations can fall below the LOD due to concentration factors that are part of the extraction protocol. Thus limit of quantification (LOQ) (3xLOD) is used for milk samples and LOD is used for all other biological media, where sample concentration is not required. The LOD in serum and the LOQ in milk are shown in Table 1.

2.5. Biological marker analysis

Selected biologics in milk and serum were analyzed for each woman according to LabCorp's standard operating procedures for these assays as previously reported in detail [39]. The assessed endpoints were in milk: Secretory immunoglobulin A, prolactin, tissue necrosis factor-α (TNF-α), interleukin-6 (IL-6), triglycerides, glucose, and estradiol; and in serum: prolactin, immunoglobins, TNF-α, IL-6, triglycerides, glucose, and estradiol. In this investigation, the milk and serum concentration of the biological markers were used to explore possible relationships with the detectable PFCs.

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Table 2
Percentage (number) of serum and milk samples with PFCs[a] > LOD at visit 1 (serum $n = 34$; milk $n = 18$) and visit 2 (serum $n = 30$; milk $n = 20$).

Perfluoroalkyl acids	Serum > LOD % (n)	Milk > LOQ % (n)
PFOS		
Visit 1	100 (34)	0
Visit 2	100 (30)	0
PFOA		
Visit 1	100 (34)	0
Visit 2	100 (30)	0
PFHxS		
Visit 1	100 (34)	0
Visit 2	100 (30)	0
PFNA		
Visit 1	97 (33)	0
Visit 2	100 (34)	0
PFOSA		
Visit 1	44 (15)	0
Visit 2	73 (22)	15 (3)
Me-PFOSA-AcOH		
Visit 1	53 (18)	5.6 (1)
Visit 2	50 (15)	0
Et-PFOSA-AcOH		
Visit 1	0	5.6 (1)
Visit 2	0	0
Perfluorobutane sulfonate		
Visit 1	[b]	0
Visit 2	[b]	0
Perfluorodecanoate		
Visit 1	[b]	0
Visit 2	[b]	0

[a] PFOS: perfluorooctane sulfonate; Et-PFOSA-AcOH: 2-(N-ethyl-perfluorooctane sulfonamido) acetic acid; Me-PFOSA-AcOH: 2-(N-methyl-perfluorooctane sulfonamido) acetic acid; PFHxS: perfluorohexane sulfonic acid; PFOS: perfluorooctanyl sulfonate; PFOA: perfluorooctanoic acid; PFNA: perfluorononanoic acid.
[b] Denotes not measured in serum.

2.6. Statistical analysis

We calculated the percentage of detection for each analyte in serum and milk, and determined the median, range, mean, standard error, and selected percentiles. For values below the LOD, values equal to LOD/sqr2 were used [48,49]. Further analyses, including relationships between visits and across media, were conducted for those analytes for which the frequency of detection (>LOD) was ≥60% at both visits. For those women who donated 2 serum samples, the median difference between the concentrations for the same PFC at visit 1 and visit 2 was calculated and assessed with the Wilcoxon signed-rank test (non-parametric). Spearman correlation coefficients and related p values were calculated for correlations between the PFCs at visit 1 and visit 2, and between the PFCs and the biological markers in milk and serum. Relations between *a priori* selected variables assessed by questionnaire and the PFCs were evaluated using Wilcoxon scores (rank sums) test. The cut-off points for categorizing selected variables were decided *a priori* based on assumptions according to data previously reported [40–42], and to achieve approximately equal distribution of numbers of subjects across categories. Two-sided p values are reported. All analyses were conducted with SAS version 9 (SAS Institute, Cary, NC).

3. Results

The median age of the women in this study was 31.3 years (interquartile range (IQR): 27.1–34.2 years), and the children's median ages were 5.5 weeks (IQR: 4–6 weeks) at visit 1 and 13 weeks (13–14 weeks) at visit 2. Three of the analytes, PFHxS, PFOS and PFOA, were detected in 100% of women's serum samples at both visits. PFNA was detectable in 97% at visit 1 and in 100% of women's samples at visit 2 (Table 2). In contrast, in milk samples of just 4 women, only 3 of the analytes were >LOQ: Et-PFOSA-AcOH (1.0 ng/ml) and Me-PFOSA-AcOH (0.7 ng/ml) were detected in 1 woman at visit 1, and PFOSA was detected in 3 women at the 2nd visit (0.3, 0.5, and 0.6 ng/ml). The remainder of the milk samples from both collections were measured and found to have concentrations < LOQ.

The distribution of PFC serum concentrations is shown in Table 3. Highest concentrations were found for PFOS with median values of 20.0 ng/ml at the first visit and 16.9 ng/ml at the second visit. PFOS concentrations were almost six-fold higher than the concentration of the analyte with the next highest value, PFOA, with median values of 3.5 and 2.9 ng/ml at the first and second visit, respectively.

Median serum concentrations were significantly ($p \leq 0.01$) lower for PFOS, PFOA and PFHxS assessed at visit 2 compared to the concentration assessed at visit 1, based on samples of 30 women who donated serum samples at both visits with the differences shown in Table 4. Accordingly, the concentrations of the detected serum PFCs are reported for each visit (Table 3). Serum concentrations of the same PFC were significantly correlated between the two visits (Table 4). Due to the limited number of breast milk samples with detectable PFC concentrations, we could not calculate the

Table 3
Distribution (mean, standard error, median, selected percentiles, IQR) of PFCs[a] in serum samples at visit 1 ($n = 34$) and visit 2 ($n = 30$) in ng/ml.

	Mean (SEM)	10th percentile	25th percentile	Median	75th percentile	90th percentile	95th percentile	IQR
PFOS								
Visit 1	21.9 (1.9)	11.7	13.2	20.0	30.1	37.6	45.7	16.9
Visit 2	18.8 (1.5)	9.70	14.0	16.9	22.6	30.2	35.5	8.60
PFOA								
Visit 1	3.99 (0.35)	1.50	2.20	3.50	4.60	6.0	8.70	2.40
Visit 2	3.0 (0.21)	1.45	2.40	2.90	3.70	4.65	5.0	1.30
PFHxS								
Visit 1	1.94 (0.27)	0.70	1.0	1.55	2.40	3.40	3.80	1.40
Visit 2	1.50 (0.22)	0.50	0.70	1.15	1.70	2.90	4.60	1.00
PFNA								
Visit 1	1.22 (0.12)	0.40	0.70	1.10	1.60	2.00	2.70	0.90
Visit 2	1.33 (0.09)	0.75	1.00	1.20	1.50	1.90	2.40	0.50
PFOSA								
Visit 1	0.07(0.01)	<LOD	<LOD	<LOD	0.10	0.10	0.10	0.07
Visit 2	0.09 (0.01)	<LOD	<LOD	0.10	0.10	0.15	0.20	0.07
Me-PFOSA-AcOH								
Visit 1	0.23 (0.02)	<LOD	<LOD	0.20	0.30	0.30	0.40	0.16
Visit 2	0.24 (0.02)	<LOD	<LOD	0.17	0.30	0.40	0.50	0.16

[a] PFOS: Perfluorooctane sulfonate; Et-PFOSA-AcOH: 2-(N-ethyl-perfluorooctane sulfonamido) acetic acid; Me-PFOSA-AcOH. 2-(N-methyl-perfluorooctane sulfonamido) acetic acid; PFHxS: perfluorohexane sulfonic acid; PFOS: perfluorooctanyl sulfonate; PFOA: perfluorooctanoic acid; PFNA: perfluorononanoic acid. Values measured <LOD were imputed by LOD/sqr2.

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Table 4
Difference and correlation in PFC serum concentrations (ng/ml) between visit one and visit two.

	Median difference (IQR)	p value*	Correlation coefficient σ*	p value
PFOS	−2.30 (−7.9, 1.0)	<0.01	0.82	<0.001
PFOA	−0.55 (−1.40, 0.0)	<0.001	0.82	<0.001
PFHxS	−0.40 (−0.80, −0.10)	<0.001	0.87	<0.001
PFNA	0.11 (−0.20, 0.50)	0.10	0.71	<0.001

* Wilcoxon signed-rank test (non-parametric) ($n = 30$).
* Spearman correlation coefficient σ and related p value ($n = 30$).

partition coefficient from serum to milk, but can conclude that milk concentrations were notably lower than serum concentrations.

Based on self-reported data, women had lived in North Carolina for (mean, SEM) 14.6 (1.92) years. Interestingly, women who had reported living in North Carolina for 10 years or more compared to those who had reported living in North Carolina less than 10 years, had higher serum concentrations of PFNA, PFOA, and PFOS ($p < 0.03$) (Fig. 1). Furthermore, living in a house with an enclosed garage attached as compared to living in a house with no enclosed garage attached, suggested a relation to higher concentrations of PFHxS (ng/ml; median, IQR, visit 1: 2.2 (1.4) *vs.* 1.1 (0.6), $p < 0.001$; visit 2: 1.5 (1.4) *vs.* 0.9 (0.7) $p = 0.03$) and of PFOS (visit 1: 25.4 (16.9) *vs.* 14.4 (9.9), $p = 0.01$; visit 2: 21.2 (11.5) *vs.* 14.5 (7.8) $p = 0.1$).

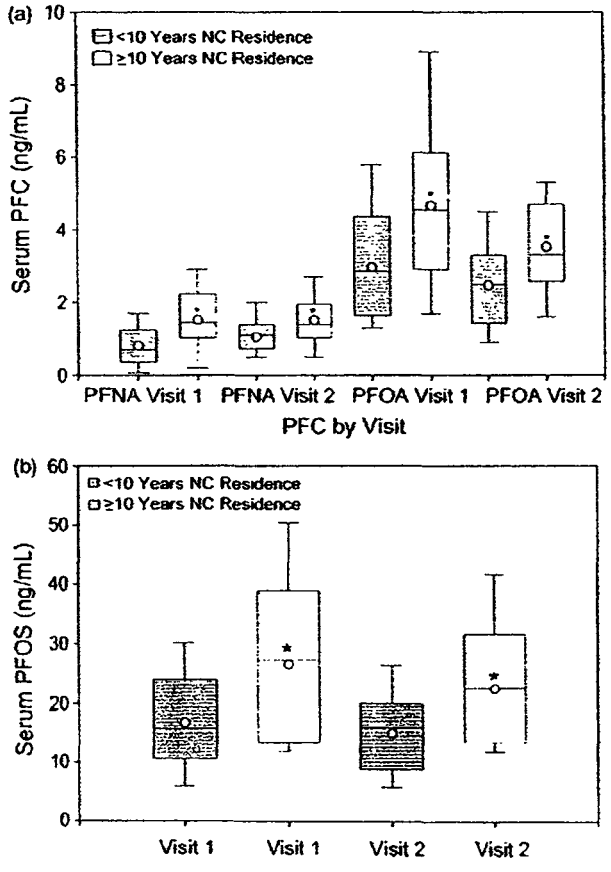

Fig. 1. (a) and (b) Serum concentrations of PFNA, PFOA, and PFOS (ng/ml) comparing living in North Carolina ≥ 10 to < 10 years at visit 1 and visit 2. Data are shown as box and whisker representations; open circles denote mean values with the medians denoted as a straight line. * $p < 0.03$ in Wilcoxon Scores (rank sums) tests for groups ≥10 years vs. <10 years at visit 1 and visit 2 for each PFC. Numbers of subjects in each group: ≥10 years: $n = 16$, visit 1; $n = 15$, visit 2; <10 years: $n = 18$, visit 1, $n = 15$, visit 2.

Table 5
Correlations between concentration of PFC and interleukin-6 in serum at visit 1 ($n = 34$) and 2 ($n = 30$).

	Correlation coefficient, σ*	p value
PFOS		
Visit 1	−0.21	0.20
Visit 2	0.39	0.03
PFOA		
Visit 1	−0.15	0.40
Visit 2	0.07	0.70
PFHxS		
Visit 1	−0.11	0.50
Visit 2	0.38	0.04
PFNA		
Visit 1	−0.003	1.0
Visit 2	−0.08	0.70

* Spearman correlation coefficient σ and related p value. Bolded values signify significant correlations.

Serum concentrations of IL-6 were positively correlated with PFOS ($p = 0.03$) and PFHxS ($p = 0.04$) at the second visit (Table 5). None of the other selected biological markers in serum or in milk showed a significant correlation with the PFC serum concentrations at either collection time point. There was no significant relationship between maternal age or parity and PFC serum concentrations in our study (data not shown). Due to the small numbers and lack of racial diversity in this pilot study based on convenience sampling (only 3 women reported themselves as Black/African-American, one as Asian and one as Hispanic), we could not analyze PFC concentrations by ethnic group.

4. Discussion

In this pilot study of healthy lactating North Carolina women, 6 of the 7 PFCs analyzed in serum were detectable at 2–7 weeks and 3–4 months postpartum. PFOS, PFOA, PFNA, and PFHxS were found in nearly 100% of the serum samples. PFOS, followed by PFOA and PFHxS were the compounds detected at the highest concentrations. Only a small proportion of milk samples had detectable values of 3 of the 9 PFCs analyzed in milk. Interestingly, serum levels were lower for PFOS, PFOA, and PFHxS at the second visit compared to the first visit, and prolonged time lived in North Carolina, as well as living in a home with enclosed garage attached, suggested a relation to elevated serum concentrations of certain PFCs in our sample; however, these analyses were unadjusted and based on a small non-random sample in this pilot study and thus should be considered exploratory. We can conclude that postnatal exposure to PFCs via breast milk is likely to be low during the time period captured in our investigation.

Data on PFC serum concentrations of lactating women are sparse and based on small sample sizes. Available data relevant for pre- and postnatal exposures to PFCs are summarized in Table 6. Only one earlier study assessed both serum and milk levels, in 12 lactating women in Sweden, and reported similar serum values to ours for PFOS (median: 18.7 ng/ml) and PFOA (3.8 ng/ml) while concentrations of PFHxS were higher (4.0 ng/ml) in the Swedish study [35]. Based on data from the Danish National Birth Cohort, prenatal maternal serum concentrations appeared to be higher for PFOS and PFOA in Denmark than seen postpartum in our study. Interestingly, in the Danish study, concentrations were lower in the second than in the first trimester, possibly due to dilution of the PFCs with blood volume expansion due to pregnancy, but values in cord blood ($n = 50$) confirmed fetal exposures [24,33] (Table 6). The serum PFC concentrations seen in our study compare well with US serum data from NHANES 2003–2004, assessed in representative samples of

Table 6
Published data on average PFC concentrations in milk, maternal serum and cord blood.

Location, year of sampling	Matrix, study population, and sample size	PFC concentration as reported	Percentage quantified > LOD[a]	Reference
Massachusetts, USA, 2004	Milk: convenience sample, age: 22–43 years, $n = 45$, nursing the first time: $n = 34$, nursed >1: $n = 8$	PFOS (mean, SD): 131 (103) pg/ml PFOA: 43.8 (33.1) pg/ml PFHxS: 14.5 (13.7) pg/ml PFNA: 7.26 (4.70) pg/ml PFHpA, PFDA, PFUnDA, PFDoDA, PFBS: all <LOD	PFOS: 96% PFOA: 89% PFHxS: 51% PFNA: 64% PFHpA, PFDA, PFUnDA, PFDoDA, PFBS: ≤8%	[25]
Leipzig/Munich Germany, 2006	Milk: convenience sampling at hospital samples, $n = 19$ (Munich)	PFOS (median, range)	PFOS: 100%	[36]
Gyor, Hungary 1996/97	Milk bank, $n = 38$ (Leipzig) Mothers of preterm infants, $n = 13$ (Hungary) at 3–7 weeks postpartum	Munich: 113 (28–239) ng/L Leipzig: 123 (33–309) ng/L Hungary: 330 (96–639) ng/L PFOA, all: <LOD (<LOD–460) ng/L	PFOA: 16%	
Zhousan, China, 2004	Milk: convenience sampling at hospital volunteers, $n = 19$	Ranges[b] (ng/L) PFOS: 45–360; PFOA: 47–210; PFHxS: 4–100; PFNA: 6.3–62; PFDA: 3.8–15; PFUnDA: 7.6–56	PFOS, PFOA, PFHxS, PFNA, PFDA, PFUnDA: 100%	[34]
Sweden, individual matched sera and milk (2004); pooled composite milk samples (1996–2004),	Milk and serum: convenience sample primiparous women, $n = 12$ Pooled annual composite milk samples ($n = 25$–90) Date of milk collection: 3 weeks postpartum	Milk (mean, SD) ng/ml: PFOS: 0.201 (0.117); PFHxS: 0.085 (0.047); PFOSA: 0.013 (0.009); PFNA: NA; PFOA, PFDA, PFUnDA: ND Serum (mean, SD) ng/ml: PFOS: 20.7 (10.5); PFHxS: 4.7 (2.9); PFOSA: 0.24 (0.16); PFNA: 0.80 (0.55); PFOA: 3.8 (1.0); PFDA: 0.53 (0.41); PFUnDA: 0.40 (0.35) Composite milk ng/ml: PFOS 0.209 (1996)–0.123 (2004); PFHxS: 0.037 (1996)–0.016 (2004); PFOSA: <0.007 (1996)–<0.007 (2004); PFNA: 0.028 (1996)–0.020 (2004); PFOA: <0.209 (1996)–<0.209 (2004)	Milk: PFOS, PFHxS: 100% ($n = 12$); PFOSA: 67% ($n = 8$), PFNA: 16% ($n = 2$); PFOA: 8% ($n = 1$) Serum: PFOS, PFHxS, PFOA, PFNA, PFDA, PFUnDA: 100% ($n = 12$); PFOSA: 75% ($n = 9$)	[35]
Baltimore, MD, USA, 2004–2005	Cord blood, hospital based, singleton deliveries ($n = 293$)	PFOA (median, range): 1.6 (0.3–7.1) PFOS (median, range) ng/ml: 5.0 (<LOD (=0.2)–34.8)	PFOS: 99% PFOA: 100%; Et-PFOSA-AcOH, Me-PFOSA-AcOH, PFBuS, PFHpA, PFUA, PFDoA: 1–40%	[22,23]
Denmark, 1996–2004	Maternal plasma: 1st trimester ($n = 1399$), 2nd trimester ($n = 200$) Cord blood, $n = 50$	Maternal, 1st trimester: PFOS (ng/ml, mean, SD): 35.3 (13.0), PFOA: 5.6 (2.5) Maternal, 2nd trimester: PFOS: 29.9 (11.0); PFOA: 4.5 (1.9) Cord blood: PFOS: 11.0 (4.7); PFOA: 3.7 (3.4)	Maternal, 1st trimester: PFOS: 100%, PFOA: 100% (except $n = 1$)	[24]
Japan, 2003	Maternal plasma: 3rd trimester ($n = 15$) cord blood ($n = 15$)	Maternal, 3rd trimester serum range[b]: PFOS (4.9–17.6 ng/ml), PFOA (<LOD to 2.3 ng/ml), PFOSA (<LOD to <LOD) Cord blood: PFOS (1.6–5.3 ng/ml), PFOA (<LOD to <LOD), PFOSA (<LOD to <LOD)	Maternal, 3rd trimester: PFOS: 100%, PFOA: 20%, PFOSA: 0% Cord blood: PFOS: 100%, PFOA: 0%, PFOSA: 0%	[30]

[a] PFC LODs for serum, blood and milk varied in the different studies as reported in the original references.
[b] No averages reported by authors.

females aged 12 and above, showing median concentrations for PFOS and PFOA of 18.2 ng/ml (IQR: 12.4–27.3 ng/ml) and 3.6 ng/ml (IQR: 2.5–5.2 ng/ml), respectively [20,21]. Based on the NHANES data, nation-wide serum concentrations dropped for PFOS, PFOA and for PFHxS between 1999/2000 and 2003/2004 while those for PFNA increased in the same time period [20]. Our average levels are somewhat lower than reported for females in the US in 1989 [5] but similar to other findings in samples collected between 1999 and 2005 [6,10,20,21,50]. In a recent US investigation, median cord blood levels for PFOS and PFOA of 5 and 1.6 ng/ml, respectively, were reported [23]. This is about a third to a fourth (PFOS) and 50% (PFOA) of the concentrations we found in maternal serum samples, and also about half the concentration reported for cord blood from Denmark [24], Table 6.

A few investigations of PFCs in human milk have been conducted in Sweden, China, Denmark and recently in the US (Table 6) [25,34–36]. Only one study assessed both serum and milk concentrations and detected PFOS and PFHxS in all 12 milk samples at mean concentrations of 0.201 and 0.085 ng/ml respectively, suggesting partitioning of on average 1% from serum to milk [35]. In the Chinese study, values of PFOS and PFOA in milk samples ($n = 19$) were in the range of 0.045–0.36 and 0.047–0.21 ng/ml, respectively [34]. Milk concentrations are summarized in Table 6, supporting our findings of lower values in milk than in serum, as well as

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suggesting regional differences in exposure levels [25,34–36]. PFAAs are strongly bound to the protein fraction of human blood [10,51–53]. The protein concentration in human blood contains mainly albumin and fewer beta-lipoproteins and is about 3–5 times higher than the protein fraction in human milk (casein and lactalbumin). It has been shown that strongly protein-bound drugs are less likely to transfer to human milk than small non-ionic lipophilic compounds [54]. This may explain why PFAA concentrations are much lower in human milk than in maternal serum, although transfer of PFAAs to milk has been observed in animal studies, albeit at much higher serum concentrations of PFAAs [26].

In our study, concentrations of PFOS, PFOA and PFHxS in serum were lower at the second visit compared to concentrations at the first visit. Since PFC concentrations measured in human sera have half-lives ranging between 3.4 years for PFOA, 4.6 years for PFOS, and 7.1 years for PFHxS [55], these data suggest that processes related to depuration into breast milk might be occurring that we could not assess (possibly because we measured milk concentrations too late in lactation), or that there might be maternal metabolic changes during lactation that may relate to this change (i.e., changes in blood volume, body weight, or hepatic activities). Because PFCs are tightly bound to serum proteins, serum protein levels during lactation could have affected the concentrations of PFCs in serum. Unfortunately, we did not measure serum albumin to test this hypothesis. Alternatively, if PFCs partition more into liver than serum in the course of lactation, serum concentrations of PFCs could be affected as well. The possible transfer of PFCs to milk may also vary at different times during lactation. The nature of the relationship between the suggested decline of PFC values in serum to concentrations in milk are yet unclear and insufficient data exist to date to explain the relationship at this point. Few earlier reports suggested declines in breast milk during lactation for lipophilic compounds including dioxins, PCBs, and PBDEs [56,57]. No other study to our knowledge, has investigated PFC concentration changes in serum or milk over time during lactation assessed in the same women at two time points. However, it should be noted that our findings are based on a relatively small number of a volunteer non-random sample of women and need replication in a larger study for further confirmation. Tao et al. conducted a regression analysis of PFOS and PFOA concentrations in breast milk collected at various time points from 25 different women within the first 6 months postpartum; they concluded that values increased over time of lactation [25]. However, since these findings were based on milk samples of different subjects rather than comparing changes over time in the same women, the differences may be due to intra-individual variation.

Our investigation suggested that living in North Carolina for a prolonged time period of 10 years and more was related to higher serum concentrations of PFNA, PFOA, and PFOS in our pilot study. However, further evaluation of this explorative finding is required. Point sources may lead to elevated exposures, as indicated by serum concentrations of PFOA in persons living near a US facility using and producing this compound, that were notably higher than among the general US population [58]. A systematic surface water survey conducted in North Carolina showed large variation in concentration on a small scale indicating a series of source inputs around the Cape Fear Drainage Basin that may potentially result in pockets with increased exposures [40]. Comparing serum PFC concentrations among donors at the 6 American Red Cross Blood Bank locations across the US showed highest concentrations for PFOS and second highest for PFOA in Charlotte, North Carolina, in samples collected in 2000–2001 [41], with a substantial decline observed in samples collected in 2006 at the same locations [42]. Recently, elevated plasma concentrations especially of PFOA, PFNA, and PFHxS have been reported for personnel involved in the World Trade Center (WTC) disaster (i.e., from fire-fighting foams used to combat the WTC fire or directly from the WTC's degradation) [59] further supporting the notion of source related local variations of human exposures to certain PFCs. Women who reported living in a home with an enclosed garage attached also had increased concentrations of PFHxS and PFOS in our sample. This may be due to certain materials used in and around cars containing PFCs, such as post-market applications of external and internal surface car coatings or treatments. However, due to the small sample size in this pilot study, we could not analyze the impacts of other variables, especially socio-economic factors; these findings are thus explorative and should be interpreted cautiously.

The pro-inflammatory cytokine IL-6 was positively correlated to PFOS and PFHxS, respectively, at the second collection time point possibly indicating that certain PFAAs may be related to inflammatory processes. In line with these findings are recent results from experimental studies in mice, reporting suppression of immune responses following exposure to PFOS *in utero* [60]. We did not see correlations with other *a priori* selected biological markers assessed in milk or serum, i.e., immunoglobulin, estradiol, prolactin or TNF-α. Rodent studies using PFOA in concentrations orders of magnitude higher than MAMA serum concentrations have shown a suppression of genetic markers of inflammation after an acute exposure to PFOA [61]. Because our findings are explorative, future studies may want to address the role of chronic exposure to low dose PFCs in the inflammatory process.

In this pilot study the number of women was relatively small and confines the investigation of associations, possible exposure pathways, and time trends after birth. In addition, the sample was not selected randomly, thus selection bias cannot be excluded. However, the participation of women was unlikely to be related to PFC exposures or to certain PFC exposure sources since they were most likely not aware of their PFC exposures. A further limitation of our study is that we could not collect milk samples sooner after birth in view of ethical constraints in asking for the colostrum milk. Studies in mice measuring PFOA concentration over the course of lactation have shown that the peak in milk PFOA concentration occurs soon after birth (Fenton et al., in this issue), a time that was not followed in the MAMA collection scheme. Overall, the findings reported are explorative and need further evaluation.

In conclusion, although infant exposure via breast milk is likely to be low, the cumulative daily infant intake of PFCs via breast milk per kg body weight could be appreciable for some populations or groups (Table 6). Since toxicological and pharmacokinetic data for PFC exposed infants are lacking, it is largely unknown if potential health effects in infants or during childhood may be related to current exposure levels of PFCs. *In utero* exposure should continue to be a concern as the MAMA serum PFAA concentrations are similar to values reported in two separate studies that have shown inverse associations between maternal serum or cord blood PFAA concentrations and infant birth weight [22,24]. Thus, the findings of this pilot study underscore the importance of biomonitoring maternal and infant exposure to PFC as well as the need for further study of the potential human health effects of PFCs. In the upcoming US National Children's Study [38] PFC exposures in pregnant and lactating women and their children in North Carolina and across the US will be further studied.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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mental Protection Agency (EPA), and the Centers for Disease Control and Prevention (CDC) and approved for publication. Approval does not signify this report reflects EPA or CDC policy. The findings in this report are those of the authors and do not reflect the views of the CDC. The use of trade names or commercial products does not constitute endorsement or recommendation for use.

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Phenotypic dichotomy following developmental exposure to perfluorooctanoic acid (PFOA) in female CD-1 mice: Low doses induce elevated serum leptin and insulin, and overweight in mid-life☆

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ABSTRACT

The synthetic surfactant, perfluorooctanoic acid (PFOA) is a proven developmental toxicant in mice, causing pregnancy loss, increased neonatal mortality, delayed eye opening, and abnormal mammary gland growth in animals exposed during fetal life. PFOA is found in the sera and tissues of wildlife and humans throughout the world, but is especially high in the sera of children compared to adults. These studies in CD-1 mice aim to determine the latent health effects of PFOA following: (1) an *in utero* exposure, (2) an *in utero* exposure followed by ovariectomy (ovx), or (3) exposure as an adult. Mice were exposed to 0, 0.01, 0.1, 0.3, 1, 3, or 5 mg PFOA/kg BW for 17 days of pregnancy or as young adults. Body weight was reduced in the highest doses on postnatal day (PND) 1 and at weaning. However, the lowest exposures (0.01–0.3 mg/kg) significantly increased body weight, and serum insulin and leptin (0.01–0.1 mg/kg) in mid-life after developmental exposure. PFOA exposure combined with ovx caused no additional increase in mid-life body weight. At 18 months of age, the effects of *in utero* PFOA exposure on body weight were no longer detected. White adipose tissue and spleen weights were decreased at high doses of PFOA in intact developmentally exposed mice, and spleen weight was reduced in PFOA-exposed ovx mice. Brown adipose tissue weight was significantly increased in both ovx and intact mice at high PFOA doses. Liver weight was unaffected in late life by these exposure paradigms. Finally, there was no effect of adult exposure to PFOA on body weight. These studies demonstrate an important window of exposure for low-dose effects of PFOA on body weight gain, as well as leptin and insulin concentrations in mid-life, at a lowest observed effect level of 0.01 mg PFOA/kg BW. The mode of action of these effects and its relevance to human health remain to be explored.

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Abbreviations: ANOVA, analysis of variance; BMI, body mass index; BW, body weight; C8, eight-carbon; CV, coefficient of variation; DES, dietylstilbestrol; E_2, estradiol; GD, gestational day; $t_{1/2}$, Half-life; IACUC, Institutional Animal Care and Use Committee; LH, luteinizing hormone; LOD, limit of detection; LOQ, limit of quantitation; NHANES, National Health and Nutrition Examination Survey; NMR, nuclear magnetic resonance; NOAEL, no observable adverse effect level; ovx, ovariectomized; PFAA, perfluoroalkyl acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonate; PND, postnatal day; PPAR, peroxisome proliferator-activated receptors; SMR, standardized mortality ratio.

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1. Introduction

Perfluorooctanoic acid (PFOA), one of the eight carbon (C8) perfluoroalkyl acids (PFAAs), is a synthetic, stable, persistent organic fluorine surfactant, used to impart water and grease resistance to various consumer products including non-stick pans, as surface treatments for clothing and food wrappers, insulation and fire-fighting foams. PFOA's high energy carbon–fluorine bonds are resistant to hydrolysis, photolysis and metabolism and thus it bioaccumulates and persists within biota and environmental matrices, including water and soil, from the Arctic to the South Pacific (Lau et al., 2007). This ubiquitous environmental contaminant has an estimated half-life ($t_{1/2}$) in humans of 3.8 years (Olsen et al., 2007) and is found in production workers' sera, as well as those of the general population.

Bio-monitoring studies show detectable levels of PFOA in human populations. The National Health and Nutrition Examination Survey (NHANES) reported that mean serum PFOA concentrations are declining in the USA population, from 5.2 ng/ml in 1999–2000 to 3.9 ng/ml, in 2003–2004 (Calafat et al., 2007). Arnsberg, Germany, an area with known drinking water PFAA contamination, had reported PFOA mean serum levels in 2006 of 25 ng/ml vs. 4 ng/ml in unaffected German provinces (Hölzer et al., 2008). The highest known non-occupational PFOA exposure via drinking water exists in the Little Hocking drinking water district where U.S. residents (Ohio and West Virginia) have mean serum PFOA concentrations of 478 ng/ml (Emmett et al., 2006).

Children may receive significant PFOA exposures via dietary and water intake. Mean serum PFAA concentrations (such as perfluorohexane sulfonic acid) were reportedly higher in children than in adult/elderly populations (Olsen et al., 2004). In the Little Hocking water district, an area of high environmental PFOA exposure, children age two to five and the elderly had significantly increased PFOA serum levels when compared with other age groups (Emmett et al., 2006). Although a bio-monitoring study in Japan found PFOA in maternal blood, but not umbilical cord blood at parturition (Inoue et al., 2004, limit of quantitation [LOQ] 35.2 ng/ml), a recent U.S. study (Apelberg et al., 2007) of human cord blood from term pregnancies reported relatively low levels of PFOA (limit of detection [LOD] 0.2 ng/ml) and another C8 compound, perfluorooctane sulfonate (PFOS). Within the reported study concentrations, the authors found that cord blood PFOA concentrations were significantly negatively associated with birth weight. A subsequent larger Danish study also found a significant negative correlation between maternal plasma PFOA and birth weight (Fei et al., 2007).

There have been no consistent adverse health effects associated with occupational exposure to PFOA, in fact, the studies to date are contradictory. In worker populations, serum cholesterol and triglycerides have been positively associated with PFOA exposure while high density lipoproteins have been negatively associated with PFOA (Olsen et al., 2001). Categorical division of workers by PFOA exposure levels showed that, although not significantly different from the other categories, body mass index (BMI) was elevated in the highest PFOA category (>30 ppm and BMIs >28, 1995 data); this trend was not seen in the 1993 data set (Olsen et al., 1998). A retrospective cohort mortality study ($n > 6000$) of PFOA-exposed employees reported significantly elevated standardized mortality ratios (SMR) in males with diabetes mellitus when compared to men residing in West Virginia (minus the PFOA manufacturing area), Ohio, Virginia, Kentucky, Indiana, Pennsylvania, Tennessee, or North Carolina; the SMR for PFOA workers was not significantly increased when compared to West Virginia alone or USA residents (DuPont, 2006). In Arnsberg, Germany, PFOA was found to have an inverse correlation with BMI in adults (Hölzer et al., 2008).

The $t_{1/2}$s for PFOA in men and women are similar (Harada et al., 2005). Unlike humans, gender differences in PFOA clearance exist in rats (Kudo and Kawashima, 2003; Vanden Heuvel et al., 1991). Mice are the preferred animal model for evaluating the effects of PFOA on the developing fetus as they do not exhibit gender-dependent $t_{1/2}$ differences (Lau et al., 2006). However, even in the rat model system where the female rat rapidly excretes the compound, PFOA readily crosses the placenta (Hinderliter et al., 2005) and PFAAs are present in rat milk after PFOA treatment (Hinderliter et al., 2005).

Mice prenatally exposed to doses of PFOA at ≥1 mg/kg/day exhibit developmental toxicity including decreased litter size, neonatal death, delayed eye opening, growth deficits, stunted mammary gland development, and early onset male puberty (Lau et al., 2006; White et al., 2007; Wolf et al., 2007). At higher doses and following long-term adult exposure, cancer endpoints associated with PFOA exposure in rats include Leydig cell adenomas, pancreatic acinar cell adenoma/carcinomas, mammary fibroadenomas, and liver tumors (Biegel et al., 2001; Sibinski, 1987). PFOA increased estradiol (E_2) levels in male rats and PFOA-induced rodent Leydig cell tumors are hypothesized to arise from increased estradiol levels from aromatase induction (Liu et al., 1996; Biegel et al., 2001).

The majority of the ongoing work in the PFOA field has focused on the health effects following developmental exposure to PFOA. This study focuses on adult latent health outcomes in female offspring after developmental (gestational days (GD) 1–17) vs. adult (at 8 weeks of age, for 17 days) exposure to PFOA. Ovariectomized siblings were utilized in our second study block to address the role of the ovarian hormones in PFOA exposure-related health effects, as luteinizing hormone (LH)-overexpressing mice (Kero et al., 2003) displayed several phenotypic effects resembling those in our preliminary studies with PFOA. These studies address the role of developmental exposure and ovarian hormones in adult health effects including circulating leptin and insulin concentrations, adult body weight, and tissue and body weights in old age.

2. Materials and methods

2.1. Animals

Timed-pregnant CD-1 mice (Charles River Laboratories, Raleigh, NC) arrived on gestational day (GD)0 (sperm positive) at the US EPA where they were weighed upon arrival and randomly distributed among treatment groups. Pregnant dams were housed individually in polypropylene cages and received chow (LabDiet 5001, PMI Nutrition International LLC, Brentwood, MO) and tap water *ad libitum*. Two blocks of animals were used in these studies. Block 1 animals were dosed with vehicle (distilled water), 1, 3, or 5 mg PFOA/kg body weight (BW) ($n = 5$, 8, 7, and 5 dams, respectively); block 2 animals were dosed with vehicle, 0.01, 0.1, 0.3, 1, or 5 mg PFOA/kg ($n = 14$ dams in all groups except 5 mg PFOA/kg BW, which had 10 dams). PFOA exposures are shown in the text as mg PFOA/kg. Animal facilities were maintained on a 12:12-h light–dark cycle, at 20–24 °C with 40–50% relative humidity. Animals were humanely treated as approved under National Health and Environmental Effects Research Laboratory protocols in accordance with the US EPA Institutional Animal Care and Use Committee (IACUC). Sentinel mice, housed in the same room, were known to be free of ecto/endoparasites and antibodies to certain viruses for the duration of these studies.

2.2. Dosing solution and procedures

PFOA, as its ammonium salt (>98% pure), was acquired from Fluka Chemical (Steinhiem, Switzerland). PFOA dosing solution was prepared fresh daily in deionized water, and the dosing solution was administered at a volume of 10 μl/g. Mice received either water vehicle or PFOA at 0.01, 0.1, 0.3, 1, 3, or 5 mg/kg BW by oral gavage once daily over the dosing periods. The highest dose (5 mg PFOA/kg/day) was chosen because it was known to result in slightly reduced neonatal body weight gain with minimal postnatal mortality (Lau et al., 2006).

2.3. Experimental design

2.3.1. Developmental exposure/intact

Timed-pregnant CD-1 mice ($n = 7$–22 dams per dose group over two blocks) received 0, 0.01, 0.1, 0.3, 1, 3, or 5 mg/kg PFOA by oral gavage on the mornings of GD 1–17. Dams were weighed daily prior to dosing and throughout gestation. At birth, pups were individually weighed and sexed. Pups within a treatment group were pooled and randomly redistributed among the dams of their respective treatment groups, and litters were equalized to 10 pups (both genders represented). Dams

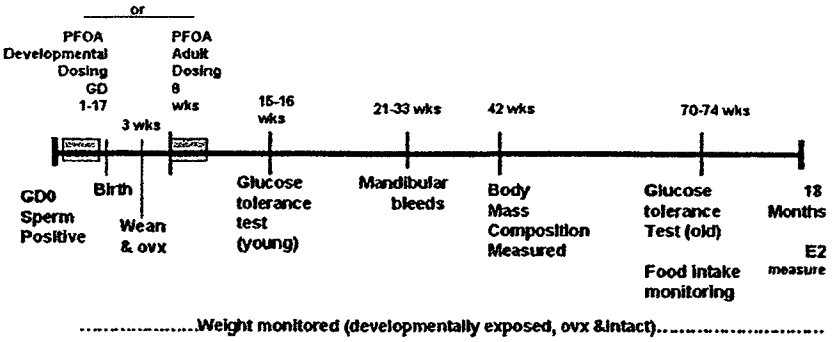

Fig. 1. Data collection schematic for study of developmentally and adult PFOA-exposed female mice.

that delivered small litters ($n < 4$ pups) were excluded from the remainder of the study. Pups were weaned at 3 weeks of age at which point females were retained and housed 3–5 mice per cage. Males were evaluated separately, at end points that varied from those reported here.

2.3.2. Developmental exposure/ovariectomy

A subset of developmentally exposed female siblings (0 mg PFOA/kg, $n = 8$; 0.01 mg PFOA/kg, $n = 15$; 0.1 mg PFOA/kg, $n = 11$; 0.3 mg PFOA/kg, $n = 14$; 1 mg PFOA/kg, $n = 6$; 5 mg PFOA/kg, $n = 7$) were ovariectomized (ovx) at 21 or 22 days of age, before the onset of puberty. Animals were sedated with ketamine/xylazine (87/13 mg/kg i.p., respectively), their ovaries surgically removed through the abdomen, sutured, and animals were placed in warming cages until they regained alertness. Buprenorphine analgesic (0.05 mg/kg) was given twice daily i.m. for 48 h in 0.1 ml volume for pain relief.

2.3.3. Adult exposure

A separate cohort of mice received PFOA starting at 8 weeks of age, for 17 days (0 mg PFOA/kg, $n = 8$; 1 mg PFOA/kg, $n = 14$; 5 mg PFOA/kg, $n = 14$).

2.3.4. Data collection

The data collection scheme for these studies is shown in Fig. 1. Blood was collected from the submandibular veins of ovx and intact mice between the ages of 21 and 33 weeks. These bleeds took place between 14:00 and 18:00, and 200 μl of blood (100 μl of serum) was collected for subsequent analyses of insulin and leptin. Females in all three exposure scenarios were weighed weekly up to 9 months of age and then monthly until 18 months. The number of intact, developmentally exposed mice weighed weekly/monthly was 10, 25, 20, 11, and 32, respectively for 0, 0.01, 0.1, 0.3, and 1.0 mg PFOA/kg. If mice became moribund before the study ended, they were euthanized in compliance with the protocol approved by the US EPA IACUC (early necropsy). Date and cause of early morbidity or mortality was recorded if known. At early necropsy (collected when necessary) or at 18 months, trunk blood, retroperitoneal abdominal white (found lying ventral to the intestines and reproductive tract) and interscapular brown fat pads, abnormal growths, and organs were collected from all exposure groups. Relative organ weight is used to express organ weight as percent of total body weight. Data are reported here as mean ± SEM.

2.4. Glucose tolerance test

Glucose tolerance tests were performed on two groups of intact developmentally PFOA-exposed animals: old adults (17 months of age with 0, 0.1, 1 or 5 mg PFOA/kg; $n = 8$–13 per dose group) and young adults (15–16 weeks old with 0, 1 or 5 mg PFOA/kg; $n = 12$ per dose group). The night before the assay, fur was shaved from the lateral area of the lower leg to expose the saphenous vein and animals were fasted. The following morning, the mice were weighed and blood glucose was measured by collecting a drop of blood from each mouse via puncture of the saphenous vein (or tail vein if necessary). The blood drop was placed on a test strip, and inserted into the calibrated glucometer (Accuchek Advantage) for baseline glucose measurement. The mice were then injected i.p. with D-glucose solution (2 g/kg body weight from a stock solution), and blood glucose concentrations were measured at 20, 40, 60 and 120 (old mice) or 180 (young mice) minutes (±1–3 min) after the initial glucose injection.

2.5. Serum leptin

Serum (10 μl) collected by mandibular venipuncture was assayed for leptin by radio-immunoassay (Linco Research, St. Charles, MO) following the manufacturer's protocol ($n = 5$, controls; $n = 18$, 0.01; $n = 16$, 0.1; $n = 11$, 0.3; $n = 24$, 1 mg PFOA/kg). The coefficient of variation (CVs) for the standards (concentration range of 0.2–20 ng/ml) ranged from 0.1% to 8.0%. The quality control standards termed QC1 (expected range 0.6–1.3 ng/ml) and QC2 (range 1.8–3.8) had a measured concentration in these assays of 0.9 and 2.9, respectively.

2.6. Serum insulin

Sera (10 μl) collected by mandibular venipuncture were assayed for insulin by the ultra-sensitive single molecule immunoassay by Singulex (Alameda, CA) following the manufacturer's protocol ($n = 9$ control, $n = 21$, 0.01 mg PFOA/kg; $n = 16$, 0.1 mg PFOA/kg; $n = 11$, 0.3 mg PFOA/kg; $n = 31$, 1 mg PFOA/kg). Samples were analyzed using a 384-well plate format with monoclonal capture and detection antibodies on the Singulex Errena equipment. The CVs for the assay standards (range 19.5–5000 pg/ml) were from 3% to 17%. The assay LOD was 16 pg/ml. All samples were run on the same day and the interassay CV was 9.4% and 5.1% for the 29 and 1745 pg/ml quality assurance standards, respectively.

2.7. Body mass composition

Whole body mass composition was measured in live, non-sedated 42-week-old mice using the Bruker Minispec mq 7.5 LF50 Live Mouse Analyzer (The Woodlands, TX). The minispec was a benchtop 7.5 MHz time-domain nuclear magnetic resonance (NMR) analyzer, which quantified body fat, lean tissue, and free body fluid in mice. The minispec was calibrated by Bruker Optics, Inc. staff prior to animal analysis with daily validations using Bruker standards. Mice were weighed and inserted into the instrument for analysis (1–2 min/animal). Intact developmentally exposed female mice that underwent body mass composition analysis included control, 0.01, 0.1, 0.3, and 1 mg PFOA/kg ($n = 9$, 23, 20, 11, and 32, respectively) dose groups. It was not possible to perform these measures with younger mice due to equipment availability.

2.8. Measurement of E_2 in serum of intact mice at 18 months

Serum E_2 (25 μl volume) from 18-month-old mice (intact developmentally PFOA-exposed animals) was measured with time resolved fluoro-immunoassay (DELFIA Estradiol Kit, Wallac Oy, Finland) following the manufacturer's recommendation using a VICTOR²D 1420 Multilabel counter, PerkinElmer Precisely time-resolved fluorometer (PerkinElmer Life & Analytical Sciences, Shelton, CT). The CVs for the standards (concentration range of 6.81–142.5 pg/ml) ranged from 0.2% to 4.8%.

2.9. Feed consumption

Feed consumption in 17-month-old, developmentally exposed, intact female mice ($n = 6$ per dose group, 0, 0.1, 1 and 5 mg PFOA/kg) was measured in metabolic cages. Mice were allowed to acclimate to the cages for 1 week and food intake was monitored during the second week. Mice were individually housed and provided with a pre-weighed amount of powdered lab chow *ad libitum*. The remaining chow was measured at the end of the week and the total amount was subtracted from the starting amount to determine the total feed consumed for each mouse per week.

2.10. Measurement of serum PFOA

Trunk blood serum samples (~50 μl) from the female CD-1 offspring at 18-month necropsies or from mice terminated at earlier intervals because of illness were transferred to the CDC for PFOA measurement. Serum PFOA determination was performed as described in Kuklenyik et al. (2005) and White et al. (2009).

2.11 Statistics

Data were analyzed using SAS 9.1 (SAS Inc., Cary, NC). Body weight on PND1 was evaluated as litter means as these data were obtained prior to mixing litter offspring within a dose group.

Body weights at each time point were analyzed with mixed effects linear models (SAS Proc Mixed) to estimate means and standard errors and test for dose effects separately by time point. For each time point the model included dose as a fixed effect

and cage nested within dose as a random effect. Pairwise *t*-tests were calculated to test for any difference between each treatment group mean and the control group.

Repeated measures analysis of body weight data was evaluated two ways. First, weights were averaged by animal over eight 10-week intervals. This was done to decrease missing values in the data due to animal mortality in late life that was not equal across treatment, and to reduce the effect of large body weight variances later in life. This data smoothing method decreased uninformative short-term variations and also reduced the number of estimated parameters to a tractable value. A multivariate repeated measures analysis (SAS Proc GLM) was performed on these reduced data. Subsequent to a significant finding, comparisons were carried out as subtests of the overall analysis of variance (ANOVA) at specific times or doses.

Second, SAS Proc Mixed was used to perform a univariate repeated measures analysis of the weights across time up until 37 weeks (latest weight point at which no animals had died). The model estimated a separate fixed quadratic curve across time for each dose group and included a random effect for cage nested within dose. Correlation within animals was modeled with a random effect for animal nested within cage and dose in addition to an autoregressive covariance structure within each animal. In this way, the covariance matrix for each animal's measurements included a constant covariance component at all time points in addition to a component which decreased as time points grew farther apart.

Tissue weight, relative tissue weight, body composition, food consumption, and body weight measurements were analyzed using a one-way ANOVA (Dunnett's post hoc tests), with dose being the independent variable. A blocking variable was included to adjust for the group difference. No adjustment was made for multiple comparisons. Glucose tolerance was compared at individual collection times by one-way *t*-test and over time by repeated measures and area under the curve comparisons according to the trapezoidal rule. Hormone (insulin, E_2 and leptin) concentrations were analyzed using ANOVA followed by Tukey's post hoc test.

Mortality data were analyzed with product limited survival estimates; log-rank and Wilcoxon tests were used to test for differences among the treatment groups in survival across time (SAS Proc Lifetest). The level of significance for all tests was $p < 0.05$.

3. Results

3.1. Developmental exposure

3.1.1. Early and mid-life body weight effects

There were no significant differences in live pup number at birth by dose group ($p < 0.05$) and postnatal mortality was not addressed in this study as litters were equalized at birth. On postnatal day (PND) 1, the average weight of the developmentally exposed 5 mg PFOA/kg offspring was significantly less than controls (Fig. 2A); no other dose group demonstrated significant litter weight effects at PND1. At weaning, mean female body weights were still significantly decreased in the 5 mg PFOA/kg (13.9 g ± 0.8) compared to 18.4 g ± 0.4 in control untreated pups. At this time, the 1 mg PFOA/kg exposed animals were also significantly smaller than controls ($p < 0.05$; 16.4 g ± 0.3).

Time-grouped mean body weights of the female offspring over their lifetime are shown in Fig. 2B. Beginning at 10–19 weeks of age, there was an increase in weight in the 0.1 and 0.3 mg PFOA/kg groups compared to controls; by 20–29 weeks of age, females developmentally exposed to PFOA showed significant dose-dependent increases in body weight at 0.01, 0.1, and 0.3 mg PFOA/kg which extended to 40 weeks of age in the 0.01 and 0.1 mg PFOA/kg when compared with control ($p \leq 0.05$). This is specifically shown at 20–29 weeks (Fig. 2C), where the 0.01–0.3 mg PFOA/kg groups had average weights 11–15% higher than controls.

Continuous analysis of repeated measures of body weight over time demonstrated that the five dose groups were similar in intercept using a quadratic fit; however, the 0.01, 0.1 and 0.3 groups had a significantly greater week effect than control, indicating that their weights were changing at a more rapid rate than control or 1 mg/kg. This is shown in Fig. 2D for weeks 6–37 (the latest weight collection time point prior to death of any study animals). Additionally, the 0.1 mg/kg ($p = 0.056$) and 0.3 mg/kg ($p = 0.046$) groups had larger negative coefficients for week^2 (week squared), suggesting that their weights were starting to fall off more quickly at the later time points than the control groups (not shown). The estimated weight curve for the 1 mg PFOA/kg dose group was not significantly different from the control curve. Data from 5 mg PFOA/kg exposed

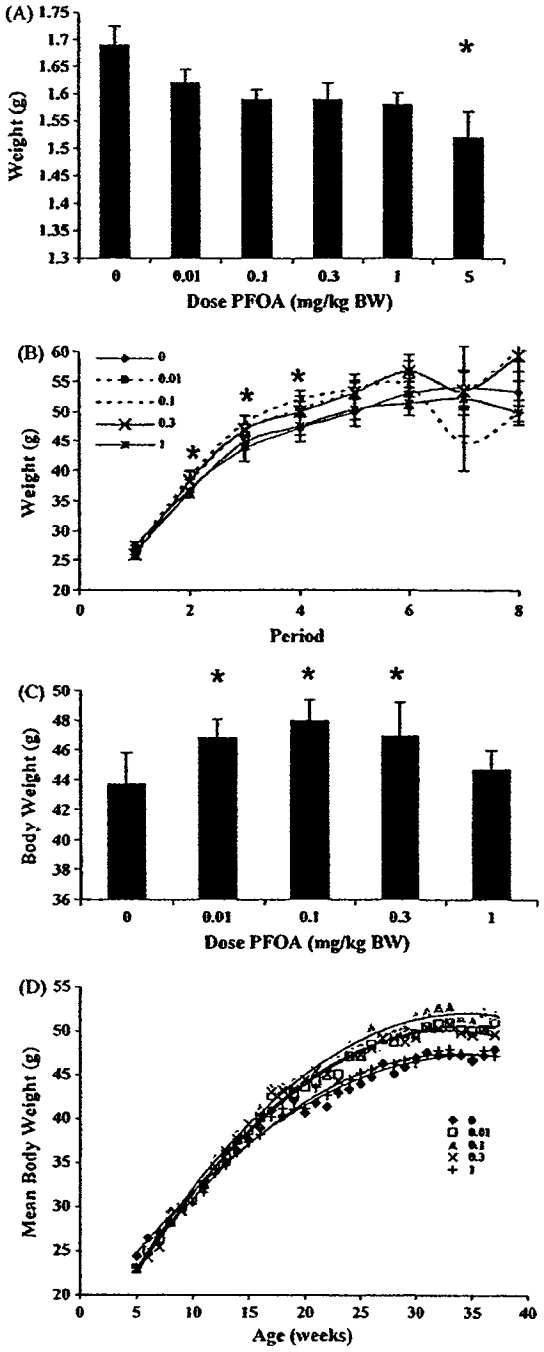

Fig. 2. Body weights of developmentally PFOA-exposed female offspring. Data are shown as mean ± SEM with $^*p < 0.05$ vs. control. (A) Pup weight at PND1 after developmental PFOA exposure. (B) Body weight of female CD-1 mice over their lifetime, following developmental PFOA exposure over 8 periods of time [period 1 (0–9 weeks old), period 2 (10–19 weeks old), period 3 (20–29 weeks old), period 4 (30–39 weeks old), period 5 (40–49 weeks old), period 6 (50–59 weeks old), period 7 (60–69 weeks old), and period 8 (70–79 weeks)]. (C) Group mean body weights of female offspring at 20–29 weeks of age demonstrating excessive weight gain at low doses. (D) Dose-dependent quadratic regression fit to repeated measures of body weight in female mice. An increased rate of weight gain was seen in 0.01, 0.1, and 0.3 mg PFOA/kg dose groups compared to control and 1 mg PFOA/kg.

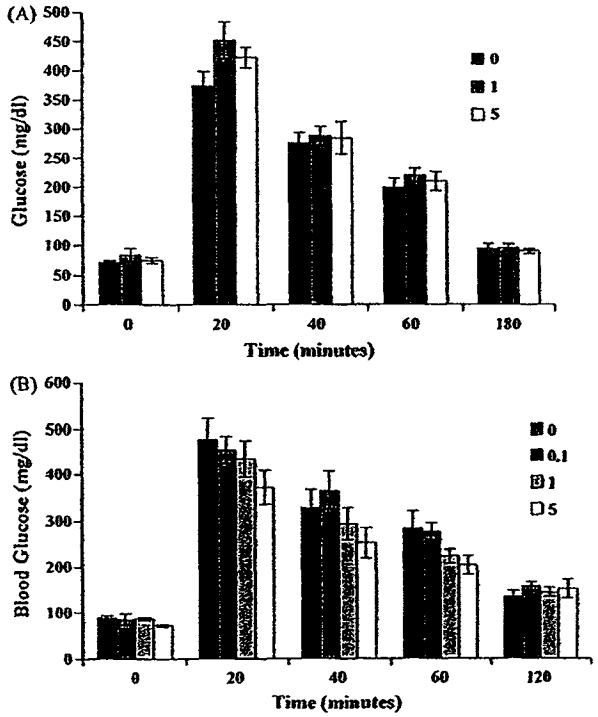

Fig. 3. Blood glucose concentrations following a glucose challenge after time 0 in (A) young (15–16 weeks old) and (B) old (70–74 weeks old) female CD-1 mice that were developmentally exposed to PFOA. Data are shown as mean ± SEM.

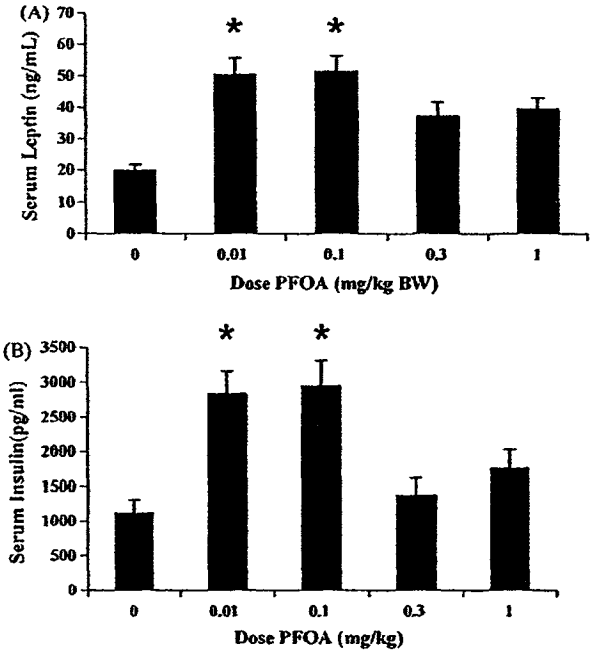

Fig. 4. Serum leptin (A) and insulin (B) in mice at 21–33 weeks of age (*$p < 0.05$ vs. control). Significant elevations are seen at 0.01 and 0.1 mg PFOA/kg. Data are shown as mean ± SEM.

mice, which were decreased in BW compared to control at PND1, weaning, and 18 months, are not shown.

3.1.2. *Serum glucose tolerance testing*

Because of the excess weight gain in the PFOA developmentally exposed mice during mid-life, various tests were conducted on these animals (as close to the appropriate age as was possible) to examine the associated effects of these changes. No significant differences were detected in baseline glucose or serum glucose area under the curve in response to a glucose challenge in young or old mice (control, 0.1, 1, or 5 mg PFOA/kg, $p < 0.05$, Fig. 3). In a time-dependent comparison, young mice exposed to 1 mg PFOA/kg showed a nearly significant increase in blood glucose over control animals at 20 min post-glucose challenge ($p = 0.06$). In old PFOA-exposed mice, although there appeared to be dose-dependent glucose insensitivity at 20 min, this shift in response was not significant.

3.1.3. *Serum insulin and leptin*

Serum insulin and leptin measurements were made using blood obtained via mandibular bleeds between 21 and 33 weeks (within the time frame of greatest observed body weight increases) using intact female mice dosed with 0, 0.01, 0.1, 0.3, and 1 mg/kg PFOA. Insulin and leptin concentrations were significantly increased in mice developmentally exposed to the lowest doses of PFOA tested (0.01 and 0.1 mg PFOA/kg). Although elevated from the control mean, leptin concentrations were not significantly different from control at 0.3 or 1 mg/kg PFOA (Fig. 4).

3.1.4. *Fat to lean ratio*

At 42 weeks of age, mice from block 2 (control, 0.01, 0.1, 0.3, and 1 mg PFOA/kg) were evaluated using a Bruker Optics Body Mass Analyzer, which determines the amount of fat, lean and fluid in live animals. There was no significant increase detected in % body fat:body weight in PFOA-exposed mice (data not shown). Developmentally exposed mice had no significant differences in fat:lean ratio across dose groups when compared to control (means ranged from 0.75% in controls to 0.9% in 0.01 and 0.1 mg PFOA/kg). Although no dose groups were significantly different from control, there was an increase above control levels of about 12% in mean % fat:body weight ratio and 14% in mean fat:lean ratio in the dose group exhibiting the largest change in body weight at 24 weeks (0.1 mg PFOA/kg).

3.1.4.1. Feed consumption. Feed consumption was measured in 17-month-old, developmentally exposed intact mice (0, 0.1, 1 and 5 mg PFOA/kg) and no significant differences were found across dose groups when compared to controls (mean 26 g/week consumed; individual data not shown).

3.1.5. *Late life organ and body weight effects*

A noted loss of animals after 36 weeks of age was further evaluated (Fig. 5). At 51 weeks old, when there was no mortality in controls there were 20%, 10%, 36%, and 6% mortality rates in 0.01, 0.1, 0.3, and 1 mg PFOA/kg groups, respectively. By 76 weeks, there was a 40% mortality rate in controls, and 32%, 63%, 60%, and 44% in 0.01, 0.1, 0.3 and 1 mg PFOA/kg groups, respectively. However, there were no significant differences between control and any treatment group at specific times in late life or in survival across time.

Among those mice surviving to 18 months, body weight of PFOA-exposed females was no longer elevated compared to controls. Furthermore, a significant decrease in body weight at the 5 mg PFOA/kg dose was noted (Table 1). At that time, all remaining females were necropsied. Trunk blood, tissues (affected or of interest) and abnormal masses were collected, weighed and fixed for future study. Serum was collected and PFOA levels were measured. The majority of the samples across dose groups had PFOA concentrations lower than the limit of detection (0.5 ng/ml) with detectable values at maximum concentrations of 3.5 ng/ml, and

Table 1
Mean or relative body and tissue weights at 18 months of age in intact and ovariectomized (ovx) female CD-1 mice.

PFOA dose (mg/kg)	Body weight (g)		Abdominal white fat		Interscapular brown		Relative spleen		Relative liver	
			Weight (g)		Fat weight (g)		Weight (%)[c]		Weight (%)[c]	
	Intact	Ovx	Intact	Ovx	Intact	Ovx	Intact	Ovx	Intact	Ovx
0	54.90 ± 1.83	52.73 ± 5.67	7.07 ± 0.56	3.83 ± 1.03	0.73 ± 0.04	0.37 ± 0.09	0.39 ± 0.05	0.52 ± 0.16	4.20 ± 0.10	4.80 ± 0.44
0.01	56.56 ± 1.48	52.61 ± 3.63	6.68 ± 0.41	5.08 ± 1.00	0.80 ± 0.04	0.36 ± 0.04	0.30 ± 0.03	0.29 ± 0.04	3.99 ± 0.11	4.12 ± 0.35
0.1	54.60 ± 1.17	52.76 ± 1.98	5.91 ± 0.37	4.84 ± 0.71	0.82 ± 0.04	0.46 ± 0.06	0.45 ± 0.12	0.40 ± 0.08	4.20 ± 0.41	4.18 ± 0.21
0.3	56.00 ± 1.74	49.36 ± 3.53	5.96 ± 0.63	4.57 ± 0.46	0.79 ± 0.06	0.39 ± 0.02	0.45 ± 0.10	0.33 ± 0.08	4.30 ± 0.31	4.07 ± 0.22
1	56.15 ± 1.35	61.47 ± 3.53	5.82 ± 0.43[a]	5.82 ± 0.77	0.89 ± 0.04[a]	0.69 ± 0.06[a]	0.30 ± 0.03	0.22 ± 0.02[b]	4.02 ± 0.10	3.55 ± 0.29[b]
3	53.69 ± 2.27	nc	nc	nc	1.22 ± 0.10[a]	nc	0.18 ± 0.03[a]	nc	3.98 ± 0.23	nc
5	49.37 ± 1.51[a]	55.13 ± 5.76	4.48 ± 0.65[a]	5.86 ± 1.67	0.86 ± 0.05	0.62 ± 0.20	0.52 ± 0.24	0.21 ± 0.04[b]	4.37 ± 0.34	3.65 ± 0.33

nc, Denotes not collected from this dose group.
[a] $p < 0.01$ vs. control.
[b] $p = 0.05–0.07$.
[c] Relative weight (organ weight as percent of body weight).

there was no significant difference in serum PFOA concentrations across dose groups (data not shown). There were no significant differences in serum estradiol levels in developmentally exposed females at 18 months when compared to controls (non-cycling; mean range across doses from 12.9 to 15.8 pg/ml).

Tissue weights from 18-month-old animals (intact and ovx) are shown in Table 1. To determine if the weight of fat depots was altered in old animals due to developmental PFOA exposures, the retroperitoneal abdominal white and interscapular brown fat pads were collected and weighed. Abdominal white fat weight and relative white fat weight both showed significant decreases vs. control ($p < 0.05$) at 1 and 5 mg PFOA/kg. White fat weights were not collected for 3 mg/kg PFOA animals. At 18 months, interscapular brown fat weight and relative brown fat weight both showed significant increases above control ($p < 0.05$) at 1 and 3 mg PFOA/kg. The spleen was quite variable in weight among the different treatment groups, but there was a significant difference in spleen weight and relative spleen weight vs. control at 3 mg PFOA/kg ($p < 0.05$). Finally, at 18 months, no significant differences in liver weight or relative liver weight were detected.

3.1.6. *Effect of ovariectomy on tissue and body weight gain*

A group of developmentally PFOA-exposed animals (0, 0.01, 0.1, 0.3, 1, and 5 mg PFOA/kg) were ovx at weaning and their body weight gain and adult health was assessed until they reached 18 months of age. At mid-life the weight of the control ovx females was expected to be greater than that of the sham-operated, intact controls (Fig. 6A; set of bars at 0 mg/kg), but the variance in the animal weights was appreciable and therefore the differences did not reach statistical significance. When comparing the body weights of animals in the ovx study by treatment group, over time (4 weeks to 18 months), using statistical methods consistent with those used for intact animals, there was no effect of PFOA (Fig. 6B). Comparison of ovx animals to intact animals at 20–29 weeks, as shown in Fig. 6A, demonstrates an absence of body weight gain over control in the ovx animals treated with PFOA. PFOA exposure did not stimulate increased weight gain (above that of control ovx) at any developmental exposure level in the absence of the ovaries (also seen in Fig. 6B). The ovx animals were siblings to the intact animals in this study.

The ovx animals were also assessed at 18 months. Developmentally PFOA-exposed ovx animals showed no significant differences in body weight when compared to control ovx females (control mean = 52.7 ± 5.67; highest mean, 1 mg PFOA/kg = 61.5 ± 3.3; Table 1).

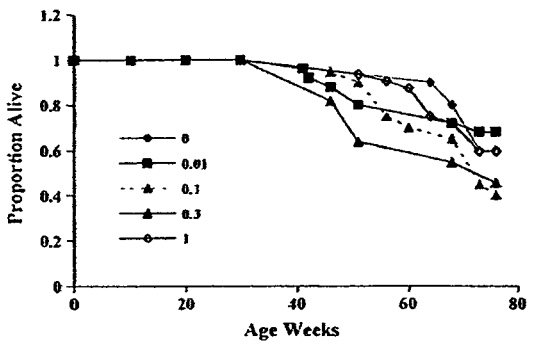

Fig. 5. Survival curves for developmentally PFOA-exposed female mice (0–1 mg PFOA/kg). Although a fair number of PFOA-exposed animals die early, a Lifetest (SAS) analysis detected no significant decrease in time to death. The reasons for early life mortality are under investigation.

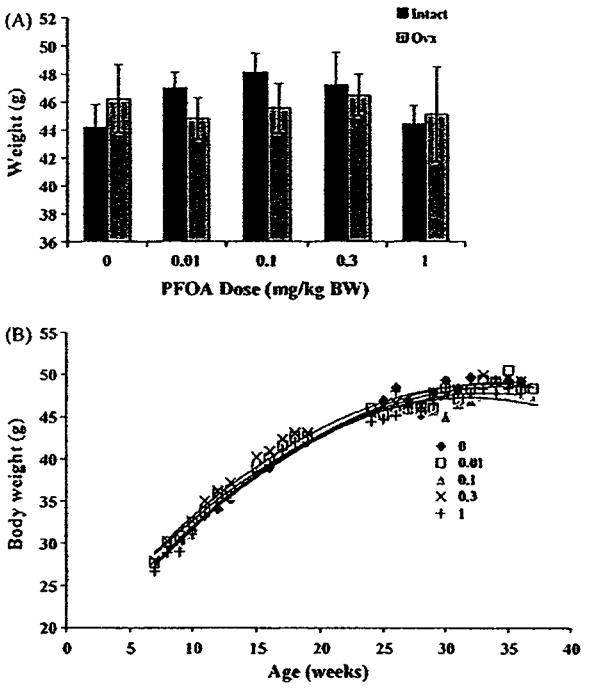

Fig. 6. (A) PFOA-dependent changes in group mean body weight of intact and ovx female offspring at 20–29 weeks of age. There was no change in body weight of ovx animals across PFOA exposures. (B) Dose-dependent quadratic regression fit to repeated measures of body weight in ovx female mice. Unlike intact siblings, no significant differences were seen between dose groups in the ovx animals.

As with intact siblings, the tissue weights of ovx animals are reported in detail in Table 1. In ovx animals, neither abdominal white fat pad weight, nor relative abdominal white fat pad weight, were significantly different from ovx or intact control levels. This varies slightly from intact siblings, where the white fat pad was significantly decreased in size; although, in animals that weighed significantly less than intact controls. Among PFOA-exposed ovx animals, both interscapular brown fat weight and relative brown fat weight (data not shown) showed significant increases above control ovx levels at 1 mg PFOA/kg ($p < 0.05$); no other dose groups showed a significant increase. This is similar to the effect seen in intact animals, and was significant at the same dose. Spleen weight (data not shown) and relative spleen weight in ovx animals was highly variable at 18 months, and showed decreases, albeit not highly significant, at the 1 and 5 mg PFOA/kg doses ($p = 0.06$ and $p = 0.05$, respectively; Table 1). 1 and 3 mg PFOA/kg (not 5 mg/kg) were the doses in the intact animals showing the largest decreases in relative spleen weight compared to controls. Finally, relative liver weight showed no significant differences across dose groups when compared to ovx control.

3.1.7. *Lack of effects from adult PFOA exposure*

At 18 months of age, body and tissue weights were recorded in adult PFOA-exposed mice. Adult PFOA exposure had no effect on terminal body or organ weights. When a comparison of data from 18-month-old adult intact and developmentally exposed animals in the 0, 1 and 5 mg PFOA/kg dose groups was made, body weight, brown fat weight, and white fat weight of the 1 mg PFOA/kg developmentally exposed animals were significantly higher than the same dose in adult-exposed animals (data not shown).

4. Discussion

These studies demonstrated the effects of developmental PFOA exposure on CD-1 female mouse body and organ weight, as well as serum leptin and insulin in adulthood. In the developmental PFOA studies, a dose-dependent dichotomy of phenotypes was present in intact female mice; latent effects present following high doses were not present in mice exposed to low-dose PFOA and *vice versa*. Although there was no detectable change in body weight neonatally, low-dose PFOA exposures (0.01, 0.1, or 0.3 mg PFOA/kg) led to significantly increased mean weight and rate of weight gain in mid-life (up to and including 37 weeks of age) and a coincident significant elevation of serum leptin and insulin values between 21 and 33 weeks (0.01 and 0.1 mg PFOA/kg).

Our low-dose hormone data indicate potentially important metabolic changes that mechanistically support the findings of increased weight in the lower dose groups. Previous dosimetry work in our lab has shown that *in utero* exposure to PFOA in the mouse translates into an extended developmental exposure period via lactational exposure (all of gestation and nearly 3 months postnatally; White et al., 2009; Wolf et al., 2007; Fenton et al., 2009). This long exposure may lead to reprogramming/metabolic events that govern fat metabolism or appetite control. Although we were unable to perform some of the other end points of interest during this time period of greatest weight gain, our findings relating leptin and insulin concentrations to the time of overweight in PFOA-exposed mice support our theory. Other environmental chemicals, termed environmental obesogens (dietylstibestrol (DES), 2OH-E_2, 4OH-E_2, genestein and bisphenol A), have been shown to induce obesity in adulthood after low-dose developmental exposure, while inducing weight loss at higher doses (Grün et al., 2006; Newbold et al., 2005; Miyawaki et al., 2007) and are reviewed further within this issue.

Serum leptin was significantly elevated in mid-life in the low-dose PFOA-exposed groups. This effect occurred at the same PFOA dose range as overweight in these animals, congruent with a leptin-resistance mechanism of action for overweight, as previously reported in humans (Considine et al., 1996). Others have reported increased leptin with developmental exposure to environmental obesogens including DES (Newbold et al., 2007).

Low-dose (0.01 and 0.1 mg PFOA/kg) developmental PFOA exposure that led to increased serum leptin and body weight also increased insulin values at 21–33 weeks. This suggests that the insulin resistance mechanistic pathway could also be affected and play a role in developmental PFOA exposure-induced overweight in mice. In an insulin resistance scenario, there are raised plasma glucose levels (elevated, but not significant, at 15–16 weeks in our study), reflecting the loss of a post-challenge peak in insulin response (reviewed in Montecucco et al., 2008). Insulin resistance is known to be associated with excess abdominal fat in normal and overweight women (Carey et al., 1996). High plasma levels of insulin and glucose, due to insulin resistance, are often associated with type II diabetes and metabolic syndrome in humans, and thus this effect of low-dose PFOA developmental exposure and its association with increased serum insulin are important.

The ovx data were difficult to interpret. The lack of additional weight gain with developmental PFOA and ovx may reflect a "ceiling effect" or that ovx-induced weight increases may have masked any effect of PFOA. Alternatively, as weight gain and metabolic hormones can be regulated by estrogens, the role of the ovaries in developmental effects of PFOA was explored by using ovx animals. The potential importance of the ovary in the effects of PFOA was based on the observation that LH-transgenic (overexpressing) mice (Kero et al., 2003) were phenotypically similar to ours (increased body weight, increased brown fat depots, and predominant ovarian cysts not discussed in this paper). We hypothesized that removal of the LH target (the ovary) in our study may reveal the mode of action for PFOA effects for the increase in brown fat and possibly the excessive weight gain. Ovx animals typically gain body weight in excess vs. intact animals (Kamei et al., 2005). The critical role of the ovary in weight gain of intact PFOA-exposed females beyond that of ovx treatment-matched siblings in the 0.01 and 0.1 mg PFOA/kg groups was novel and signifies the ovarian axis as a potential mediator of PFOA-dependent mid-life weight changes.

Another potential mediator of these intertwined low-dose PFOA-induced effects is the peroxisome proliferator-activated receptor (PPAR) activation pathway. PPAR gamma (PPAR-γ) and PPAR alpha (PPAR-α) are involved in lipid metabolism in adipocytes and liver/skeletal muscle, respectively (reviewed in Medina-Gomez et al., 2007; Abbott, 2009). These PPAR isoforms are known to influence lipogenesis/weight gain and have been shown to be regulated by environmental compounds such as tributyltin (Grün et al., 2006; reviewed in this issue). Weight loss events in leptin-deficient, obese, and insulin-resistant mouse models have coincided with PPAR-regulated changes in gene expression (Holvoet, 2008). A down-regulation of PPAR isoforms involved in energy expenditure, lipogenesis or fatty acid synthesis have been reported in adipose and skeletal muscle of ovariectomized mice (Kamei et al., 2005). PFOA has been shown to be a PPAR activator in liver tissue (high doses) and cell lines, and to be required for PFOA-induced developmental toxicity in mice (Takacs and Abbott, 2007; Abbott et al., 2007; Abbott, 2009). If PPAR activation via receptor binding is a primary mode of action for body weight effects following PFOA exposure, the decrease in the PPAR receptors following ovariectomy and decreased circulating estrogens may explain the lack of effect of PFOA in ovx mice. However, PFOA-induced consequences of PPAR activation following a developmental exposure are just beginning to be evaluated.

After 40 weeks of postnatal age, an increase in mortality was detected in all animals. There are previous reports in the literature of increased mortality in non-treated CD-1 mice, attributed primar-

ily to thymic lymphomas (Son, 2004; Taddesse-Heath et al., 2000). Because of this confounding circumstance, repeated measures of body weight were only followed out to 37 weeks of age.

The other half of the phenotypic dichotomy caused by developmental PFOA exposure was also novel. Developmental exposure to higher doses of PFOA (1, 3 and 5 mg PFOA/kg) led to a vastly different phenotype from low-dose PFOA exposure. This effective PFOA dose dichotomy may manifest itself in our study via unique modes of action; the animals with highest dose(s) of developmental PFOA exposure have decreased early life body weight and terminal body weight (5 mg PFOA/kg) with significant decreases in white fat weight at 18 months (1 and 5 mg PFOA/kg), significant increases in brown adipose (1 and 3 mg PFOA/kg), and significant decreases in spleen weight (3 mg PFOA/kg) findings that are absent with the lower doses of PFOA.

Others have reported dose-dependent loss of white tissue adiposity in adult male mice after PFOA exposure (0.02% PFOA weight/chow weight, which translated to approximately 32 mg PFOA/kg BW daily) with fat loss, without fat cell number loss, that is PPARγ-independent with β-adrenergic activation (Xie et al., 2002). In that same study, investigators also reported white fat and body weight decrements at higher doses that were absent at lower doses. Yang et al. (2002) showed PFOA-dependent weight loss was abrogated in PPAR-α null mice, indicating that PPAR-α is a probable regulator of weight loss in the high dose animals. In subsequent studies, Xie et al. (2003) showed that after cessation of exposure of adult male animals to PFOA (0.02% PFOA weight/chow weight, 32 mg PFOA/kg BW) daily for 7 days followed by 10 days recovery, weight loss and white adipose levels returned to baseline, which confirms the importance of developmental exposures for the latent effects reported here. In our model with developmental PFOA exposure we see permanent weight loss and white adipose tissue loss at the high dose of PFOA. However, there may be merit in further exploring these mechanisms of action, as β-adrenergic receptor upregulation is also associated with increased brown fat mass in winter-acclimated animals (Feist, 1983), and this tissue was associated with high dose (and not low dose) effects in both intact and ovx animals in this study. Although we suspected alleviation of effect in the brown fat pad by eliminating the ovary (based on phenotypes in Kero et al., 2003), significant increases in brown fat were seen at 1 mg PFOA/kg in both intact and ovx animals.

At the 18-month time point, some endpoints remained unchanged across dose groups including liver size. Earlier work has shown significant hepatomegaly after developmental PFOA exposure (1 and 3 mg PFOA/kg) observed out to at least 3 weeks after birth (the latest time point evaluated; Wolf et al., 2007; White et al., 2007). The transient nature of hepatomegaly has been illustrated in other acute adult exposure studies (reviewed by Lau et al., 2007), and is further confirmed in these studies (intact and ovx).

A final important component of these studies evaluated adult vs. developmental exposure to PFOA on body tissue weights. These data suggest that the timing of dosing (adult vs. developmental 17-day PFOA exposure) was critical for latent effects. There was no effect of 17-day adult PFOA exposure on any endpoint in this study (early life or latent) when compared to age-matched, vehicle-gavaged controls.

In conclusion, the timing and dose of PFOA exposure for induction of dichotomous, persistent, adult health effects in CD-1 female mice are critical. Developmental, low-dose PFOA exposure led to increased weight in adults, with increased serum insulin and leptin, a health effect not seen in high dose animals. No observable adverse effect levels (NOAEL) for body weight gain, serum leptin and insulin concentrations were not determined in this study; but 0.01 mg PFOA/kg had a significant impact on these particularly sensitive end points. The ovary appeared to play an important role in the overweight effect in mid-life, and it is proposed that there is a common mode of action, potentially dysregulation of PPAR and its signaling through ovarian hormones, that may be responsible for these low-dose health effects. Further studies addressing long-term PFOA-induced health outcomes in mice should focus attention on internal dose relative to the low-dose health effects seen in this study, as well as the mechanisms of action, so that any relevance to human health effects can be addressed.

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